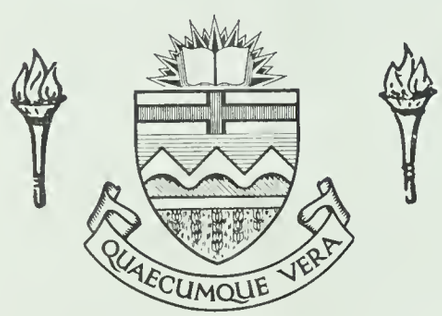

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A STUDY OF ERYTHROPOIESIS IN A GRAFT-VERSUS-HOST REACTION

by

© ERNST PINNO

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled A STUDY OF ERYTHROPOIESIS IN A GRAFT-VERSUS-HOST REACTION submitted by Ernst Pinno in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The graft-versus-host reaction in F_1 hybrid mice has been shown to be accompanied by a shift of erythropoiesis from the bone marrow to the spleen. An investigation to elucidate the nature of this shift was carried out. The reaction was induced in adult (C57L/J female x A/HeJ male) F_1 male mice by an intraperitoneal injection of 5-10 x 10^7 A/J spleen cells, or by an equal number of spleen cells from A/J mice which had been sensitized to the foreign histocompatibility antigens of the hybrid by a single intraperitoneal injection of F_1 hybrid spleen cells seven days before transfer of the A/J donor cells to the hybrid recipients. Erythropoiesis was assessed on the eighth day of the graft-versus-host reaction by measuring the plasma clearance rate and the uptake by various organs of radioactive iron.

Stimulation of the reticulo-endothelial system by the intravenous administration of colloidal carbon also produced a shift of erythropoiesis from bone marrow to spleen. However, it differed from that produced in the graft-versus-host reaction in that it was less pronounced and unaccompanied by splenomegaly and increased plasma iron turnover.

Splenectomy of hybrid mice, followed in one week by the injection of parental spleen cells, produced some amelioration of the reaction but did not abolish the erythropoietic depression in the bone marrow.

Autoradiographic stathmokinetic studies on the eighth day after spleen cell transfer revealed the following: (i) a reduction in numbers

of lymphocytes and an increase in myelocytes and metamyelocytes in both bone marrow and spleen; (ii) a decrease in the number, mitotic index and mean cell diameter of erythropoietic cells in the bone marrow; (iii) an increase in the number of erythropoietic cells in the spleen with no change in their mitotic index or mean cell diameter.

The above findings were interpreted as follows:

1. Stimulation of the reticulo-endothelial system does not account for all of the ferrokinetic and erythrokinetic alterations observed during the graft-versus host reaction.

2. Bone marrow erythropoietic depression is not strictly dependent upon concurrent changes in the spleen.

3. The observed shift of erythropoiesis from the bone marrow to the spleen is due to two factors: (a) In the bone marrow there is an arrest of proliferation of large erythropoietic cells which normally are mitotically active. (b) There is an emigration from the bone marrow of smaller, more mature erythropoietic cells, many of which settle in the spleen.

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I. INTRODUCTION

Two basic principles of tissue transplantation have been established (1). First, success or failure depends upon the degree of genetic disparity between the donor and the host. Second, graft rejection is the direct result of an immunological attack by the host against the donor. The genetic principles are based largely on the work of Little on inbred strains of mice (1, 2, 3). That allograft incompatibility is not innate but is an immunological phenomenon was demonstrated by Medawar in 1944 (4). Since that time the principles of classical immunology have been applied in the study of transplantation immunity with special emphasis on the host-versus-graft (HVG) reaction.

The immunological concept of a graft-versus-host (GVH) reaction is an outgrowth of Medawar's work. Two workers share the credit for the formulation of a GVH reaction. Simonsen (5) and Dempster (6) simultaneously published results of their work on renal allograft rejection in the dog. Histological examination of the transplanted kidney revealed large numbers of basophilic and pyroninophilic cells, presumably of the plasma cell series, in the interstitium of the renal cortex three to four days after grafting. These cell infiltrations were thought also to show transitional stages between the resting reticulo-endothelial cells of the renal interstitium and the immature plasma cells, as well as displaying mitotic figures. Both of these authors interpreted these cell infiltrations as being of donor origin actively engaged in an immunological reaction against the foreign host antigens. This simultaneous and

mutually supportive evidence established the concept of a GVH reaction and provided a stimulus for research in a new direction.

There is ample evidence today that the cell infiltrates observed by Simonsen and Dempster are primarily of host rather than donor origin (7, 8, 9, 10). Nevertheless, the GVH concept was placed on a sound basis in 1957 with evidence from two independent sources. Simonsen (11) injected 18-day chick embryos with allogeneic spleen cells and observed in the recipients splenic enlargement followed by a general lymphoid atrophy, hemolytic anemia and a positive direct Coombs' test. A similar experiment on newborn mice produced similar findings. Since chick embryos and mouse neonates are immunologically immature and hence incapable of initiating a reaction against the donor cells, Simonsen concluded that the pathological lesions observed were the direct result of the mature allogeneic donor cells reacting against the foreign antigens of the immature host. Billingham and Brent (12), working on the induction of immunological tolerance in newborn mice, observed varying mortality rates and degrees of tolerance for different strain combinations. They explained these findings on the basis of a GVH reaction with the vigour of the reaction being directly dependent upon the degree of genetic disparity between graft and host. The combined evidence provided by these two groups of workers established the validity of the GVH concept enunciated four years earlier.

Billingham (13) has stated that three conditions must be met in order for grafted cells or tissues to initiate and sustain an immunological reaction against the host: first, the graft must contain "immunologically

competent cells"; second, the host must contain important transplantation antigens which are lacking in the graft, so that the host appears foreign to it; and third, the host must itself be incapable of reacting against the graft, at least for a sufficient length of time for the latter to exert its immunological capabilities.

Simonsen (14) defined the term "immunologically competent cells" as "those cells which are capable of giving a GVH reaction". Medawar (15) described four different tests of immunological competence: (i) the restoration of immunological capability to animals deprived of it; (ii) the ability to cause splenomegaly, runt disease, or any other manifestation of GVH reactions; (iii) the ability to raise pockmarks on the chorioallantoic membrane of the fowl; (iv) the ability to raise a delayed-type inflammatory reaction in guinea pigs by the intradermal injection of normal blood lymphocytes from guinea pigs of different antigenic make-up.

The capacity to function as immunologically competent cells has been ascribed to various cell types. Terasaki (16) induced splenomegaly in chick embryos by injecting adult blood lymphocytes. As few as 30,000 lymphocytes doubled the spleen weight. Blood monocytes,cultured in vitro, were inactive in numbers up to 620,000.

Szenberg and Warner (17) found a high positive correlation between the number of foci on chick embryos and the number of large lymphocytes in the donor blood, but no dependence upon the number of small lymphocytes. They also found that large thymic lymphocytes, sometimes referred to in the literature as thymocytes, were virtually inactive.

In contrast to the above, Solomon (18) fractionated large and

small blood lymphocytes by means of a glass bead column and was able to induce splenomegaly in chick embryos with small but not large lymphocytes. Billingham and Silvers (19) grafted small pieces of adult chicken skin onto the chorioallantoic membrane and observed splenic enlargement. They ascribed this effect to donor lymphoid cells fixed in the skin.

Studies on rodents have also led to differing conclusions. Cole and Garver (20) found that sublethally irradiated F_1 hybrid mice injected with parental blood cleared of small lymphocytes by Cortone acetate still developed splenomegaly,the degree of which correlated well with the numbers of large lymphocytes injected. In direct conflict are the studies of Gowans (21) on rats which clearly demonstrated the immunological competence of small thoracic duct lymphocytes, and by assumption, the small lymphocytes of peripheral blood. Further to this, autoradiographic studies showed that at least a small proportion of the injected small lymphocytes were transformed into the large pyroninophilic cells observed in the lymph nodes during the early period of the GVH reaction. Large lymphocytes, on the other hand, were shown to be incapable of inducing GVH disease. These findings are supported by Hildeman et al. (22) who obtained 96-100% pure suspensions of small lymphocytes from the peripheral blood of mice by the use of glass wool columns to remove the granulocytes and monocytes and induced GVH disease in neonatal recipients. Simonsen (14) suggests that the lymphocyte is at least the main cell type responsible for initiation of a GVH reaction.

The inability of the host to retaliate effectively may be brought about by a number of conditions such as genetic tolerance, physiological immaturity, relative "strengths" of the antigens of the host and donor,

generalized immunological depression brought about by x-irradiation, anti-metabolites, or neonatal specifically induced tolerance to tissue antigens of the donor(23).

The simplest and purest form of the GVH reaction is produced in genetically tolerant hosts. This is the case in grafting from a homozygous donor (AA) to a host which is a first generation hybrid (F_1) between the donor strain and a different homozygote (BB), i.e. to a hybrid of the genotype AB (14). Symbolically: $A \rightarrow (A \times B)F_1$. The graft possesses no antigens foreign to the host and, hence, a HVG reaction cannot occur. Conversely, the graft can react against the BB-derived antigens of the host. This is a common form of the GVH reaction and was employed in the study to be described.

The pathological manifestations of the GVH reaction have been described in some detail by Simonsen (14) and by McBride (23). Although there are species and strain differences, the main features include growth retardation and/or emaciation, anemia, dermatitis, diarrhea, hepatomegaly, lymphoid tissue lesions, hyperphagocytic activity of the reticulo-endothelial system, and, in some strain combinations, death. Perhaps the most prominent and most constant finding in all species investigated has been the early hypertrophy of the lymphoid tissue, particularly the spleen.

Simonsen (14) has enumerated the following three factors which have been shown to affect the severity of a GVH reaction:

(i) The strength of the antigenic stimulus.

The histocompatibility antigens of the mouse have been thoroughly investigated and it was found that the H-2 locus determines the strongest immunogens (24). Hence, recipients possessing H-2 antigens absent from the

donor suffer the severest form of the reaction, all other factors being equal. A milder form of the disease is produced when host and donor differ only at loci other than the H-2. Of the fifteen histocompatability loci thus far discovered in the mouse, only the antigens of the H-2 locus invoke the synthesis of hemagglutinating and cytotoxic humoral antibodies in measurable quantities (14). The counterpart to the H-2 locus in chickens is the B blood group locus (24, 25, 26).

(ii) The number of viable cells grafted.

In general, there is a positive correlation between the number of immunologically competent cells grafted and the severity of the disease (14). In addition, the route of sensitization is also important, the order of effectiveness being intravenous>intraperitoneal>subcutaneous (12).

(iii) The age of the recipient at the time of grafting (27,28). In general, the younger the host, the more violent the attack.

The intensity of a GVH reaction has been measured in a number of ways reflecting the variable expression of the condition. A systematic account of these assay systems has been presented by Simonsen (14). Of the methods available, the spleen assay, in its various modifications, has been the most widely used. The simplest form gauges the GVH reaction by the degree of splenomegaly in the host. It has been shown by Bain (29) that peak spleen enlargement for the mouse strain combination A/J →LAF_1 (C57L/J female x A/HeJ male) occurs nine days after the parental cell injection. By the twentieth day spleen weights were again within normal range. However, it was demonstrated early in the study of GVH reactions that the onset and duration of the disease depends upon strain combination as well as age and weight of the animals (12).

Preimmunization of the donor against the host usually leads to an increase in the severity of the resulting disease (30), especially in strain combinations sharing a common H-2 locus but differing at weaker loci such as H-3 (31).

Hematological observations during the GVH reaction have been reported for a number of species. Simonsen (11) noted that chicks which were injected intravenously as 18-day embryos with spleen cells from adult donors developed a very severe anemia which began on the third post-hatching day. Washed red blood cells from the anemic animals generally gave a positive direct Coombs' test. Sera of these birds did not contain agglutinins which would react with the erythrocytes of the donor. The anemia was interpreted as being due to an immune hemolysis caused by the formation of humoral antibodies against the foreign histocompatibility antigens on the recipient erythrocytes. The normal hemopoietic structure of the marrow was gradually destroyed and replaced by the same kind of basophilic and pyroninophilic cells which were found in the spleen. The cytology of the peripheral blood was not examined.

Porter (32, 33, 34) in a series of three papers in 1960 described the GVH reaction in the rabbit. Twenty to twenty-two-day-old fetuses were injected intraperitoneally with 50×10^6 adult spleen cells. Fourteen animals died spontaneously of runt disease 50 - 70 days after birth. The bone marrows of two were normal, five were hypoplastic, three were aplastic, and four exhibited granulocytic hyperplasia. Furthermore, these rabbits also had a diminished erythrocyte survival time which was attributed to changes in host erythrocytes. These results were compatible with the view that grafted cells formed antibodies against the host red

cells causing them to be lysed. Roughly 50% of the runts gave a positive direct Coombs' test. Porter was able to elute the antibodies from the Coombs' positive cells and coat other cells with the eluate. Polychromasia and anisocytosis was noted in all animals and twelve of fourteen runts had elevated serum bilirubin levels compatible with the hemolytic nature of the anemia. Cell counts indicated a steady fall in the peripheral blood lymphocytes in all runts.

An interesting new feature of runt disease was added by Nisbet and co-workers (35, 36). These workers found that runt disease in rats could take one of two distinct forms: "pink" form and "pale" form. The pink rats had congested paws, snout and ears and copious splenic hemopoiesis, while the pale ones were anemic looking and sometimes jaundiced. Although the Coombs' test was not performed, there was "presumptive evidence of hemolytic anemia" in the pale form. A minority of the pale animals also showed an increase in the iron-containing histiocytes in the spleen and lymph nodes, as well as an increase in the plasma cells in spleen and lymph node sinusoids. The remainder of the pale runts exhibited hypoplastic marrows, polychromasia and anisocytosis. Pink runts had excessively thick blood and greater than normal red blood cell counts together with abundant splenic hemopoiesis and marrow hyperplasia. Marrow tissue sections occasionally showed collections of large, pyroninophilic cells which were thought to represent monocytoid cells rather than abnormal granulocytes.

Further hematologic observations on runt disease in rats were reported by Billingham (37). Young runted rats exhibited a pronounced

fall in the numbers of red blood cells. Marrow prints revealed hypoplasia of the erythrocytic series, with a shift of the granulocyte/erythrocyte ratio to approximately 4:1 (normal 3:2). There were striking quantitative and qualitative alterations in the white blood cells of the peripheral blood. Beginning on the seventh to eighth day there occurred a four-to ten-fold increase in the white blood cell count due mainly to granulocytes, there being no change in the lymphocyte population. The qualitative changes included a shift to the right in the Arneth count and a persistently basophilic, sparsely granulated cytoplasm of these highly lobulated cells. The marrow and peripheral blood picture was described as a hypoplastic, dyschromic anemia with absolute granulocytosis and profound alteration of the maturation of granulocytes.

Hematologic changes in mice undergoing the GVH reaction have been studied by several authors (29, 38, 39, 40). Oliner et al. (38) reported the following findings in a number of F_1 hybrid-parental strain combinations: anemia, leucopenia, lymphopenia, thrombocytopenia and reticulocytosis. The majority of animals which developed anemia also gave a positive direct Coombs' test. Eluates of Coombs' positive red cells contained antibody activity specific for the red blood cell antigens of the host inherited from the parental strain which did not serve as the lymphoid cell donor. Red cell survival studies indicated a shortened survival time of runt erythrocytes. Thus, the anemia in mice would also appear to be due to an immune hemolysis as postulated by Simonsen (11) and Porter (32, 33, 34) in chickens and rabbits respectively.

Kaplan and Rosston (39) and Harris et al. (40) differed with the

above workers in their explanation of the anemia. These authors studied the survival of Cr^{51}-labeled red blood cells in F_1 hybrid mice undergoing the GVH reaction and found a sudden loss of circulating labeled erythrocytes, whether host or parental. The conclusion reached was that the anemia is not due solely to the specific immunologic reaction of donor tissue against host erythrocytes.

One of the mouse strain combinations employed by Oliner and his co-workers (38), A/J → LAF_1, is also the combination used in the study to be described. Oliner found that LAF_1 males injected intraperitoneally with 22-150 x 10^6 female A/J spleen cells did not develop anemia, leucopenia, thrombocytopenia or reticulocytosis. If, however, cells from donors which had been preimmunized against F_1 hybrid tissue antigens (e.g. A/J sensitized with C57L spleen cells or LAF_1 erythrocytes) were used, the LAF_1 recipients developed significant hematologic abnormalities including anemia, leucopenia, lymphopenia, thrombocytopenia, granulocytosis and reticulocytosis. Thus, preimmunization is necessary to demonstrate hematologic alterations in this strain combination, in spite of the fact that the strong H-2 locus barrier is crossed in transplanting cells from A/J into LAF_1.

The effect of parental A/J spleen cells on the erythrokinetics of LAF_1 mice was investigated by Bain (29). The salient findings were increased plasma radioiron clearance, plasma iron turnover, splenic radioiron uptake and decreased radioiron uptake by the bone marrow. Cells from donors presensitized against recipient antigens produced a greater effect in each case. In addition, recipients treated with pre-

sensitized cells displayed a significantly decreased erythrocyte survival as well as a lowered hematocrit. The findings indicated an increase in total erythropoiesis in animals suffering the GVH reaction, together with a shift of erythropoietic activity from the bone marrow to the spleen. The nature of this erythropoietic shift, as it pertains to the GVH reaction in LAF_1 mice, is the subject of this thesis.

II. MATERIALS

1. Animals

The experimental animals were A/J and LAF_1 mice. The LAF_1 are the F_1 hybrid generation from matings between the inbred strains C57L/J and A/HeJ (i.e. C57L/J female x A/HeJ male). There are no known genetic differences between A/J and A/HeJ although they have been maintained as separate colonies for 41 years (41). Hereafter, these animals will be designated as LAF_1 , A and C57L for LAF_1, A/J and C57L/J respectively. All mice were obtained at five weeks of age from the Jackson Laboratory[1].

In order to avoid variations which might be introduced by the estrous cycle in the female,only males were employed in all experiments. The mice were housed in plastic or fiberglas cages with wood shaving nesting material, to a maximum of six per cage (6 in. x 9 in. x 16 in.), in a temperature-controlled (72 ± 1°F), ventilated room prior to and during the experiments. The room was constantly illuminated by fluorescent lights. Vit-a-mite[2] cubes (a vitamin-enriched blend of fishmeal, bonemeal, wheat and oats) and tap water were supplied to the animals ad libitum.

The histocompatibility genetic constitution of the above mice is presented in Table I. It will be noted that in transplanting tissues from either A or C57L into LAF_1, the H-2 barrier is crossed.

TABLE I *

HISTOCOMPATIBILITY GENETIC CONSTITUTION OF MICE

Strain	H-2 alleles	Alloantigenic specificities
A/J	a	1 - 3 4 5 6 - 8 - 10 11 13 14 - - - - 25 27 28 29 - - - -
C57L/J	b	- 2 - - 5 6 - - - - - - 14 - - - 22 - 27 28 29 - - - 33
LAF_1	a,b	1 2 3 4 5 6 - 8 - 10 11 13 14 - - - 22 25 27 28 29 - - - 33

* Adapted from Table 24-13, p. 473, Biology of the Laboratory Mouse, 2nd Ed., 1966, E. L. Green, ed., McGraw-Hill Book Company, Toronto.

A negative (-) symbol denotes the absence of the component.

2. Chemicals and Stains

a. Stains

Various nuclear stains were tested for use in conjunction with the photographic emulsion for autoradiography. The technique followed throughout these experiments was to coat Wright-stained smears with celloidin prior to application of the phographic emulsion. The Feulgen stain, applied either before the application or after the development of the photographic emulsion, produced excellent staining. It had, however, two drawbacks: (i) staining prior to the application of the photographic emulsion resulted in a loss of label from the cells, probably due to leaching of iron from the cells during the hot acid hydrolysis; (ii) staining after development of the emulsion resulted in removal of the gelatin emulsion from the smear and, hence, loss of the silver grains. Weigert's iron hematoxylin and acetic orcein also produced good staining when used alone, but gave a pale, blurred stain when applied either before coating with the photographic emulsion or after its development. The application of Wright's stain through the emulsion similarly produced poor quality staining. However, if Wright's stain was applied before the emulsion, satisfactory staining was obtained but, upon subsequent photographic development, there was non-specific grain development throughout the smear, probably due to the presence of a quinone grouping in Wright's stain (42). This difficulty was overcome by coating the stained smears with a thin film of celloidin[3] (1% celloidin in 1:1 ether-ethyl alcohol) before dipping in photographic

emulsion.

Eosin Y[4], in a 0.2% saline solution, was used to determine the number of viable cells in a marrow or spleen suspension.

b. Plasma Iron Reagents

Thioglycolic acid, trichloroacetic acid and potassium acetate required for the plasma iron determinations were obtained from the Fisher Scientific Company[5]. The color reagent, bathophenanthroline, (4, 7, diphenyl-1-10 phenanthroline) was supplied by the G. Frederick Smith Chemical Company[6]. Only reagent grade chemicals were used.

c. Radioactive Label

Radioactive iron used to tag hemoglobin-forming cells and to determine plasma iron clearance rates was obtained, in the form of a solution of $Fe^{59}Cl_3$, from Abbott Laboratories[7].

d. Photographic Emulsion

The photographic emulsion used was Kodak liquid nuclear track NTB-3[8]. This is a highly sensitive emulsion, with an average silver halide grain size of 0.2 microns, and responsive to all charged particles. Two parts of emulsion were diluted with one part of distilled water for use.

e. Stathmokinetic Agent

The metaphase-arresting drug, colchicine, employed in the stathmokinetic studies, was obtained as a 1 mg/ml solution in saline from the Laboratoires Houdé[9]. It was diluted 1:10 with 0.85% NaCl for use.

f. Colloidal Carbon

Reticulo-endothelial system stimulation was effected by the intravenous administration of Pelikan carbon black suspension C11/1431a[10]. This is a special shellac-free preparation of colloidal carbon containing 10% carbon black of particle size 200-500 A°, 4.3% fish glue as a stabilizing agent, and 1% carbolic acid as a preservative. It does not interfere with *in vivo* clotting mechanisms (43).

3. Glassware

a. Plasma Iron Determination

All glassware required for the determination of plasma iron levels was washed in a soap solution, soaked overnight in 6N HCl, and rinsed repeatedly in distilled water.

b. Handling of Cell Suspensions

In order to reduce non-specific cell death (44) during the preparation of cell suspensions, only siliconized[11] glassware was employed.

c. Autoradiographs

Autoradiographs were prepared on 25 x 75 mm Esco[12] microscope slides which had been cleansed with acid-alcohol (1 ml conc. HCl to 99 ml 95% ethyl alcohol), rinsed in 95% ethyl alcohol, and air-dried prior to use.

Footnotes

[1] The Jackson Laboratory, Bar Harbor, Maine, U.S.A.

[2] North West Feeds, Edmonton, Alberta

[3] "Parlodion", Mallinkrodt Chemical Works, Montreal, Canada

[4] Gurr's, Esbe Laboratory Supplies, Toronto, Canada

[5] Fisher Scientific Company, Edmonton, Alberta

[6] G. Frederick Smith Chemical Company, Columbus, Ohio, U.S.A.

[7] Abbott Laboratories, North Chicago, Illinois, U.S.A.

[8] Eastman Kodak Ltd., Toronto, Ontario, Canada

[9] Laboratoires Houdé, 9 Rue Dieu, Paris X^{e}, France

[10] Gunther Wagner Pelikan Werke, Hannover, Germany

[11] "Siliclad", Clay-Adams, Inc., New York, N.Y., U.S.A.

[12] Canadian Laboratory Supplies Ltd., Toronto, Ontario, Canada

III. METHODS

1. General Methodology

a. Experimental Design

In general, the test groups were of three kinds: (1) LAF_1 mice receiving spleen cells from unconditioned A/J donors (hereafter designated A → LAF_1); (ii) LAF_1 mice injected with spleen cells from A/J donors iso-immunized against the foreign histocompatibility antigens of LAF_1 (hereafter designated $A^{*LAF_1 \rightarrow LAF_1}$); (iii) LAF_1 mice injected with colloidal carbon (hereafter designated C.C. → LAF_1). The controls were LAF_1 mice receiving spleen cells from syngeneic donors (hereafter designated $LAF_1 \rightarrow LAF_1$).

Not every experiment involved all four groups. In one study LAF_1 recipients were splenectomized seven days before receiving a spleen cell injection. Modified groups are set forth in greater detail in the appropriate sections.

Experiments which required serial observations, such as the determination of plasma iron turnover (PIT), were conducted by serial sacrifice of groups of mice rather than by serial blood collections from individual mice. The small size of mice and the relatively large volumes of blood required make repeated collections from individual mice undesirable because of resulting modifications of red cell mass and blood volume.

b. Preparation of Cell Suspensions

The spleen was chosen as the source of immunologically competent cells for the induction of the GVH reaction. It offers two advantages

over other sources such as the lymph nodes, peripheral blood and thoracic duct: it is readily accessible and it yields large numbers of cells.

Generally, spleen cell suspensions were prepared as follows: The donor mouse was lightly anesthetized by ether inhalation, decapitated with a pair of scissors, and allowed to exsanguinate to reduce the residual blood in the spleen. Using aseptic technique, a midline incision was made, the spleen dissected free, placed in a sterile, siliconized Petri dish containing 1.0 ml of cold (4°C), sterile 0.85% NaCl, and teased apart with the edge of a sterile, 50 gauge stainless steel screen. The coarse stroma was sifted out with the screen and the remaining fine clumps dispersed by repeated gentle aspiration and expulsion from a tuberculin syringe fitted with a 22 gauge needle. The viability of cells in such suspensions was determined to be 85% - 90% according to their ability to exclude eosin Y. Routinely, cell counts on the suspensions were performed in a hemocytometer chamber of standard dimensions (Hawksley, England) using 2% acetic acid as diluent.

c. Induction of the GVH Reaction

Throughout this study, the GVH reaction was induced by the injection of parental spleen cells into the peritoneal cavities of mature F_1 hybrids. According to Billingham and Brent (13) the intraperitoneal route is the second most effective method of inducing a GVH reaction. It was chosen over the intravenous route since large numbers of cells may be administered without fear of causing circulatory distress.

i. Unsensitized Donors

Eight-to ten-week-old normal A mice served as donors. They were sacrificed by decapitation and spleen cell suspensions prepared as described above. Immediately after the spleen cell preparation, LAF_1 recipients were lightly anesthetized with ether and given an intraperitoneal injection of the freshly prepared A cells using a tuberculin syringe with a 22 gauge needle. The dose was normally one-half spleen in 0.5 ml saline corresponding to 5-10 x 10^7 cells. Most of the experimental observations were made eight days after spleen cell transfer when, as indicated by a previous investigation, the GVH reaction is near its peak (45).

ii. Presensitized Donors

Certain of the A donors were presensitized to the foreign histocompatibility antigens of the LAF_1 by a single intraperitoneal injection of LAF_1 spleen cells (one-half spleen dose, 5-10 x 10^7 cells). Seven days later these immunized A mice served as spleen cell donors to LAF_1 recipients. This method of sensitization results in a typical second-set reaction to LAF_1 skin grafts (29). Simonsen (31) has stated two reasons for not using foreign homozygous cells (i.e. C57L in this case) as the source of immunizing antigen. Firstly, they might possibly survive in the prospective donor's spleen long enough to give a GVH reaction after injection into the F_1 hybrid. Secondly, they might produce in the A donor a GVH reaction which might interfere with the process of immunization (46, 47).

d. Preparation of Fe^{59}-Transferrin Complex

To simulate physiological conditions, radioactive iron for intravenous injection was bound to the transport protein, transferrin, in vitro. This was achieved by incubating fresh, normal LAF_1 serum with $Fe^{59}Cl_3$ for one hour, with occasional agitation, in a 37° C incubator. The concentration of Fe^{59} for ferrokinetic studies was 2.5 µc/ml and for autoradiographic purposes 16.67 µc/ml.

2. Ferrokinetic Studies

a. Procedure

Forty-four A → LAF_1, 44 C.C. → LAF_1 and 44 LAF_1 → LAF_1 mice were utilized. LAF_1 mice which were to receive colloidal carbon intravenously were placed into a plexiglass cage under the beam of a desk lamp for 10-15 minutes immediately before the injections to dilate the veins and thereby facilitate the injections. After removal from the heat, these animals were transferred to a perforated plexiglass restraining cage and given a dose of 16 mg carbon/100 gm body weight in a lateral tail vein with a 30 gauge needle fitted to a tuberculin syringe.

On the seventh or eighth day after the spleen cell (intraperitoneal) or carbon (intravenous) injections, the animals were warmed under a desk lamp as described above and given intravenous injections of the serum-Fe^{59} complex (2.5 µc/ml), prepared as previously described, at a dosage of 0.5 µc Fe^{59}/20 gm body weight in a volume of 0.20-0.30 ml. Counting standards were prepared by making 1:50 dilutions of aliquots of the injection solution with 0.85% NaCl. Groups of four animals were

sacrificed serially by decapitation, 15 minutes to 96 hours after the Fe^{59} adminstration. Samples of blood and serum were collected for radiocounting and serum iron determinations. The right femur and the spleen were dissected free of adhering tissue and placed in tubes containing 1 ml of 10% buffered neutral formalin for radiocounting and fixation.

The radioactivity levels in the spleens, femurs, blood and serum samples at various times after the Fe^{59} injections were determined in a NaI well-type scintillation counter (Nuclear Chicago, 2½ inch NaI [Tl activated] crystal, with pulse height analyzer).

Serum iron levels were determined by a modification of a micro-method described by Forman (48). One hundred and fifty µl (0.15 ml) of serum was employed and the volumes of reagents adjusted proportionately. Trial runs on control sera indicated that the optical density was unaltered by the ethanol-chloroform extraction. Accordingly, this step was omitted. Optical densities were measured in a Coleman Jr. spectrophotometer, Model 6C, with the aid of a micro-cuvette adapter.

Hematocrit values were determined as follows: 75 mm heparinized capillary tubes, having an outside diameter of 1.3 to 1.5 mm (Clay Adams, Incorporated, New York), were three-quarters filled with the mixed venous and arterial blood obtained by decapitation, centrifuged for four minutes in an International Microcapillary centrifuge, Model MB, and read on a circular model reader.

b. Calculations

The uptakes by the spleens and femurs were calculated as per-

centages of the injected radioactivity and plotted as a function of time.

Formulae applied in computing serum and erythrocyte radioactivity were:

i. Correction of hematocrit (Hct) for trapped plasma = observed Hct x 0.95.

ii. Counts/minute (cpm)/mm^3 whole blood = $\frac{cpm/mg}{1.056}$.

iii. Cpm/mm^3 serum = $\frac{cpm/mg}{1.026}$.

1.056 and 1.026 are the specific gravities of blood and serum respectively. They are used to convert weight measurements to volume measurements (49).

iv. Blood volume in mm^3 = body weight in mg x $\frac{6.95}{100}$.

6.95 is the blood volume expressed as a percentage of body weight (49).

v. RBC cpm/mm^3 whole blood = cpm/mm^3 whole blood-[(1-corr. Hct) x cpm/mm^3 serum].

vi. Total RBC cpm = RBC cpm/mm^3 whole blood x blood volume in mm^3.

vii. Serum volume in mm^3 = Blood volume in mm^3 x [1-Hct (0.95)].

viii. Total serum cpm = serum volume in mm^3 x cpm/mm^3 serum.

Plasma radioactivity was plotted as a function of time on semi-logarithmic paper. The clearance curves for the three groups of mice were each resolved into three components by backward subtraction from (i) the actual points, and (ii) from smoothed curves drawn by inspection. The backward subtraction technique for actual points was as follows:

(i) the group of observed points judged to comprise the third component was extrapolated to time zero; (ii) the extrapolated values of the third component were then subtracted from the observed values at the corresponding times, producing a new set of points representing a two-component curve; (iii) the points judged to comprise the second component of the new two-component curve were extrapolated to time zero; this line represents the second component of the original clearance curve; (iv) the extrapolated values were subtracted from the remaining points resulting in a final linear set of points representing the first component of the clearance curve. Smoothed curves were resolved in like manner except that the subtractions were made from imaginary points on the curve which may or may not correspond to actual observations.

The method of backward subtraction uncovers the initial clearance function which is believed to represent clearance to a labile erythropoietic pool (50, 51). The $T_{\frac{1}{2}}$ for each component was estimated graphically. The fraction of iron removed from the serum per hour was calculated from the $T_{\frac{1}{2}}$ of the first component by the formula $\frac{0.693}{T_{\frac{1}{2}} \text{ (hours)}}$. The PIT was calculated as follows:

$$\text{PIT (mg/day)} = \begin{matrix}\text{Fraction of iron} \\ \text{removed/hour}\end{matrix} \times \begin{matrix}\text{Serum iron} \\ \text{(mg/ml)}\end{matrix} \times \frac{A}{100} \times \begin{matrix}\text{Plasma vol.} \\ \text{(ml)}\end{matrix} \times 24$$

where A = zero intercept of first component.

3. Effect of Splenectomy

a. Surgical Procedure

Anesthesia was induced in 18 LAF_1 mice by the intraperitoneal

injection of Nembutal (0.625 mg/10 gm body weight). The fur of the left abdominal area was removed with an electric hair clipper, the area cleansed with isopropyl alcohol and an incision of 1 - 1¼ inch made. The spleen was exposed and then separated from adhering tissue by electro-desiccation with a Hyfrecator (The Birtcher Corporation, Los Angeles, California) and discarded. The incision was closed with #4-0 silk sutures. Blood loss during the entire operation was negligible. Eighteen LAF_1 controls were sham-operated in an attempt to cause an equivalent amount of surgical trauma.

b. Experimental Groups

Seven days after splenectomy or sham-splenectomy the animals were treated as follows:

i. Nine splenectomized LAF_1 received one-half spleen doses of A cells (A → splen. LAF_1) and nine received syngeneic cells (LAF_1 → splen. LAF_1).

ii. Nine of the sham-splenectomized LAF_1 were given one-half spleen doses of A cells (A → sham-splen. LAF_1) and the remaining nine received LAF_1 cells (LAF_1 → sham-splen. LAF_1).

Eight days after the spleen cell transfers (15 days post-splenectomy) the animals were given an intravenous injection of 0.5 μc Fe^{59}/20 gm body weight. Exactly six hours later they were sacrificed to determine the radioactivity levels in the spleen (where applicable), right femur, circulating erythrocytes and serum as previously described (page 21). In addition, the right upper lobe of the liver and a one-inch segment of tail was assayed for radioactivity content.

4. Autoradiographic Stathmokinetic Studies

a. Pilot Experiments

i. Colchicine as a Metaphase-Arresting Agent

In order to test the metaphase-arresting capacity of the colchicine solution employed, four 9-week-old normal C57L male mice were injected intraperitoneally with the recommended dosage of 2 μg colchicine/gm body weight (0.45-0.50 ml of the diluted solution, p. 15) (52). Two control animals were given an equivalent volume of saline. Two test animals and one control were killed by decapitation three hours after receiving colchicine or saline respectively and the remaining three mice three hours later (i.e. six hours after colchicine or saline). Both femurs were removed, their epiphyses cut off and the marrow extruded into 1.0 ml of cold 10% bovine serum albumin in saline with a tuberculin syringe and 22 gauge needle. The cell suspension was centrifuged at 300 g for five minutes, the sediment resuspended in 1.0 ml of 1% sodium citrate and the tube allowed to stand at room temperature for five minutes. Following this, the suspension was recentrifuged, under the same conditions as previously, and the sediment suspended in two drops of normal mouse serum. Using a Reeve's #2 squirrel's hair brush, the cells were brushed onto precleaned 25 mm x 75 mm microscope slides. The smears were rapidly air-dried and then stained with Wright's stain. Each slide was scored for the number of mitotic figures per 2000 nucleated marrow cells. An average of 3.20% and 5.30% of cells were arrested at metaphase after three and six hours respectively. Corresponding control values are 0.20% and 0.25% (Table II). The somewhat diminished mitotic activity observed during the

TABLE II

METAPHASE-ARRESTING CAPACITY OF INTRAPERITONEALLY ADMINISTERED COLCHICINE

	Group	Time after colchicine or saline (hours)	Mitotic figures per 2000 nucleated marrow cells	% of cells in mitosis	
Control	1	3	4	0.20)	
				)	Mean 0.22
	2	6	5	0.25)	
Test	1	3	57	2.85)	
				)	Mean 3.25
	2	3	73	3.65)	
	3	6	108	5.40)	
				)	Mean 5.30
	4	6	104	5.20)	

second 3-hour period might indicate that some of the cells arrested early have escaped the colchicine arrest (52, 53) or it might simply be due to diurnal variation. The nature of the data and the numbers of animals were such that statistical tests of significance could not be applied. It was concluded that colchicine in the dose and manner applied is effective in arresting mitosis.

ii. Cell Expansion

Difficulty was experienced in the recognition of mitotic figures in Wright-stained autoradiographs. This was due partly to the presence of thickened, and in some cases, fused chromosome strands in the colchicine-blocked metaphases (54), and partly to heavy labeling over some cells. This difficulty, it was postulated, could be overcome by subjecting cells to a hypotonic solution which would (1) expand the cells and spread the chromosomes, thereby making the mitotic figures more easily distinguishable, and (2) not leach out the Fe^{59} label. To determine the optimal strength of the hypotonic solution with these characteristics, samples of spleen and marrow cells were exposed to graded concentrations of sodium citrate (1% - 2.5%) for 15 minutes in a 37°C water bath prior to being brushed onto slides for staining and autoradiography. Based on microscopical evaluation of autoradiographs, expansion with 2% sodium citrate was selected as combining satisfactory labeling density with ease in the identification of mitoses. Some comparative data on bone marrow cells incubated in 10% bovine serum albumin (BSA) in 0.85% NaCl (an eutonic medium), 1.25% sodium citrate and 2% sodium citrate is shown in Table III. It will be noted that the use of 1.25% sodium citrate led to complete

TABLE III

SOME DATA ON THE EFFECT OF SUSPENSION MEDIA ON THE FRACTION OF CELLS LABELED AND THE MITOTIC INDEX OF BONE MARROW CELLS

	Suspension Media		
	10% BSA (Labeling Control)	2.0% Sodium Citrate	1.25% Sodium Citrate (Mitosis Control)
Total number of nucleated marrow cells evaluated	1000	1012	1021
Number of unlabeled cells in interphase	860	863	976
Number of labeled cells* in interphase	109	101	0
Number of labeled cells in mitosis	6	12	0
Number of unlabeled cells in mitosis	25	36	45
Fraction of cells labeled	0.115	0.111	0.000
Mitotic index (M.I.)	0.031	0.047	0.044

*Maximum background was taken as the mean grain count calculated from 200 segmented neutrophils and eosinophils plus 3 standard deviations. Any cell with a grain count greater than the maximum calculated background was considered labeled.

loss of the radioactive label, whereas 10% BSA-treated cells appear to have a diminished mitotic activity, probably as a result of failure to identify a few of the mitotic cells as such.

b. Mitotic Arrest and Cell Labeling

The rationale for the combined usage of colchicine and Fe^{59} was as follows: Colchicine arrests mitosis at the metaphase stage resulting in an accumulation of metaphases; Fe^{59} specifically labels those cells actively synthesizing hemoglobin. Given these two substances in combination, the percentage of "unlabeled" cells and of "Fe^{59}-incorporating" or "labeled" cells entering division during a given period of time may be ascertained. From this data, the time required for complete turnover of these two cell "compartments" can be calculated.

This study involved two major groups of animals: 24 $A^{*LAF_1 \rightarrow LAF_1}$ mice divided into four subgroups each consisting of six animals, and 24 $LAF_1 \rightarrow LAF_1$ mice also divided into four 6-member subgroups. On the eighth day of the GVH reaction one subgroup (six animals) of $A^{*LAF_1 \rightarrow LAF_1}$ and one subgroup of $LAF_1 \rightarrow LAF_1$ mice were warmed under a desk lamp, anesthetized with ether, and given an intraperitoneal injection of colchicine (2 µg/gm body weight). This was immediately followed by an intravenous injection of radioiron (5 µc Fe^{59}/mouse in 0.3 ml). Six hours later these animals were sacrificed by decapitation and the second subgroup of both test and control animals was injected, and so on until an entire 24-hour period was covered. In this manner possible diurnal variations of mitotic activity are included.

c. Cell Suspensions and Smear Preparation

As each animal was sacrificed, the spleen and both femurs were removed. Spleen weights were determined on an analytical balance and recorded as fresh, wet weights. The marrow of both femurs was washed out as described on page 26 and pooled in 2.0 ml of cold (4°C) 10% BSA. The spleen was also suspended in 2.0 ml of BSA using a stainless steel screen as described previously. After performing nucleated cell counts on each suspension, it was divided into two 1.0 ml portions in microcentrifuge tubes and centrifuged at 300 g for 10 minutes. The supernatants were discarded and to one tube of each suspension was added 1.0 ml of 10% BSA and to the other 1.0 ml of 2% sodium citrate. The contents of all tubes were thoroughly mixed, the tubes were placed in a 37°C water bath for 15 minutes and then recentrifuged at 300 g for 10 minutes. To the pellet of cells remaining in each tube after the supernatant was removed, two drops of normal mouse serum were added, the cells resuspended and brush smears made as has already been described (page 26). All slides were stained with Wright's stain and then dipped twice in a 1% celloidin solution (page 14), allowing an interval of 10-15 minutes.

d. Autoradiography

Under a Wratten Series 2 safelight, Kodak Nuclear Track emulsion, NTB-3, was liquefied in a 45°C water bath. Two parts of emulsion were mixed with one part distilled water in a plastic slide holder, which was held at 45°C for the dipping. The Wright-stained, celloidin-coated slides were dipped, one at a time, drained, air-dried

for 20-30 minutes and placed in individual lead containers in light-tight bacteriological jars (B.T.L. Anaerobic jar, Baird & Tatlock (London) Ltd., England). The air in these jars was replaced with CO_2 gas and the jars stored at 4°C.

After 21 days, the slides were developed by the following process:

(i) Two minutes in Vividol, 1:1 dilution with water, at 68 ± 1°F.

(ii) Fifteen seconds in 2% short stop (acetic acid).

(iii) Four to five minutes in hypo (acid fixative).

(iv) Washed in gently flowing water at 68 ± 1°F.

All chemicals were from General Aniline Films (Canada) Ltd., Cooksville, Ontario, Canada.

e. Grain Counting and Cell Differential

Autoradiographs were examined under oil immersion at a magnification of 1600 X. All data collected, with the exception of cell measurements, refers to cells incubated with 2% sodium citrate. As a further check, however, BSA streaks were also evaluated and the degree of labeling compared to that of citrate streaks. In no case was there significant loss of label from citrate-treated cells as indicated by the percentage of cells considered labeled.

The background grain count was determined in a manner similar to that of Odartchenko et al. (55). The number of grains directly overlying a cell was recorded for a minimum of 500 consecutive nucleated cells. The number of grains over eosinophils, segmented neutrophils and

neutrophilic band cells was summed and the mean grain count calculated. These cells were chosen for two reasons: (i) they do not incorporate iron and hence should exhibit only non-specific, background grains, and (ii) they are easily identifiable. Any cell with a grain count greater than the calculated maximum background (Table III) was considered to be labeled. It must be emphasized that only grains directly above a cell were included in the count. However, with the isotope employed, scatter is a feature. Hence, the grain count over a cell tends to be less than the true grain count, whereas the background tends to be elevated.

A minimum of 500 nucleated spleen cells and 500 nucleated bone marrow cells were examined per animal. Cells of the granulocytic series were subdivided into myelocytes, metamyelocytes, band neutrophils, segmented neutrophils, eosinophils and lymphocytes by the usual morphological criteria. Erythrocytic cells were recorded as "labeled" nucleated cells, polychromatic erythrocytes or mature red blood cells. Lymphocytes were distinguished from normoblasts by the absence of labeling. Large immature cell forms were classified as blast cells. Mitotic figures were reported as labeled or unlabeled with no attempt being made to assign them on morphological grounds to a particular cell type within the erythrocytic or granulocytic lineage respectively.

f. Determination of Cell Size.

The diameters of labeled nucleated cells in the marrow and spleen were measured using a calibrated ocular micrometer grid. Four spleen and four marrow smears of each animal group were selected at random,

one from each 6-hour period, and the diameters of fifty labeled cells measured in each.

g. Calculations

The following terms were employed as defined below:

(i) Mitotic Index (M.I.) - Fraction of cells of a "compartment", "pool" or "cell renewal system" in mitosis (56). To facilitate calculations, mitotic indices in the present study are expressed as percentages rather than fractions (i.e. M.I. x 100).

(ii) Turnover time (T.T.) - Time required for division of 100% of cells within the population (52).

The relationship between turnover time and mitotic index expressed as above is:

$$\text{T.T. (hours)} = \frac{100\%}{\text{24 hr. M.I. (\%)}} \times 24 \text{ hrs.}$$

The "compartments" or "cell renewal systems" investigated were the "labeled cell compartment" and the "unlabeled cell compartment".

IV. FERROKINETIC STUDIES IN LAF_1 MICE TREATED WITH PARENTAL SPLEEN CELLS OR COLLOIDAL CARBON

The effects of a variety of substances and conditions on the erythropoietic system of the rodent have been studied by several authors (29, 57-64). In general, the observation common to these studies was the prominent role of the spleen. In contrast to the human spleen, the murine spleen is erythropoietically active in adult life even under physiological conditions (65), and may become the primary site of erythropoiesis following a variety of stimuli (62, 64, 66, 67). One of the interesting concomitants of splenic erythropoiesis has been a depression of marrow activity in the following conditions: (i) administration of zymosan (61); (ii) starvation and re-feeding (62); (iii) pregnancy in mice (63); (iv) GVH reaction (29). A feature common to these conditions is stimulation of the reticulo-endothelial system (RES) (68-71). This observation led to the study described below in which ferrokinetic alterations following the administration of colloidal carbon were examined and compared with those in the GVH reaction.

1. RES Stimulation

It was reported by Kelly *et al.* in 1960 (70) that the intravenous administration of 5 mg of colloidal carbon into Swiss mice resulted, 2 - 17 days later, in a two- to three-fold increase in liver DNA synthesis as measured by the uptake of P^{32}. Furthermore, autoradiographs of liver sections, prepared four hours after injection of tritiated thymidine, showed that littoral cells were mainly responsible for the

elevated DNA synthesis. Taken together, this evidence indicated that colloidal carbon stimulates RE cell proliferation. Other substances, too, were shown to possess this capacity to a lesser or greater extent (70). Among them, in order of increasing effectiveness, were iron oxide, bacterial endotoxin, zymosan and Estradiol. In addition, these substances, with the exception of iron oxide, also stimulated the functional capacity of the RES as determined by the intravascular clearance of colloidal carbon.

In the present study, colloidal carbon was utilized as an RES stimulating agent. The shellac-free carbon suspension, C11/143la, containing 100 mg carbon black per ml of suspension, was diluted to 16 mg per ml with sterile 0.85% NaCl for use.

2. Plasma Iron Turnover

The peregrinations of intravenously injected radioiron can be traced by appropriate serial sampling of the peripheral blood and/or external monitoring. In general, the route of transferrin-bound radioiron is as follows: plasma → bone marrow → circulating red blood cells (72). The rate at which iron is cleared from the plasma for incorporation into the erythroid cells of the bone marrow is regulated by body requirements, being rapid in the presence of anemia and slow in iron overload (73). Plasma iron clearance curves may be resolved into several components of which the first and most rapid has been postulated to represent clearance to a labile erythropoietic pool (50,51). The reduced clearance following the initial rapid rate has been taken as evidence for a feedback of radioiron into the plasma (55, 73).

Pollycover and Mortimer in 1961 (51) interpreted the plasma

radioiron clearance as the sum of three exponential components:

$X = Ae^{-r_1 t} + Be^{-r_2 t} + Ce^{-r_3 t}$ where

X = concentration of radioiron in compartment X

A, B, and C = compartments contributing to X

r_1, r_2, r_3 = rates of iron removal from A, B, and C respectively

e = base of natural logarithm

t = time

Generally, pools B and C have been ignored in calculating PIT (51, 73-75). According to Bothwell et al. (73) this produces an overestimation of the iron turnover. In the studies herein described Pollycove's formula was applied to the ferrokinetics of mice with appropriate corrections being made for components B and C.

3. Results

Fe^{59} uptakes by the spleen and femur are shown in Figures 1 and 2 and Table IV. Recipients of colloidal carbon exhibited an increase in erythropoiesis in the spleen and a decrease in the bone marrow as compared with control ($LAF_1 \rightarrow LAF_1$) animals but the degree of this shift was not as great as in animals undergoing the GVH reaction due to the injection of parental cells. Peak spleen uptakes occurred 5 - 12 hours, and peak femur uptakes 3 hours after injection of radioiron. The peak mean spleen and femur uptakes and standard deviations for each group of mice are presented in Table IV. Statistical analysis by Student's t-test shows a significant increase in peak spleen Fe^{59} uptake and a significantly decreased marrow uptake for both GVH animals and colloidal carbon-treated animals as compared with control mice. Table IV further

Figure 1. Radioiron uptakes by spleens of A → LAF_1, C.C. → LAF_1 and LAF_1 → LAF_1 mice. Each point is the average value of 3 - 5 mice.

Figure 2. Radioiron uptakes by femurs of A → LAF_1, C.C. → LAF_1 and LAF_1 → LAF_1 mice. Each point is the average of 3 - 5 mice.

Figure 1.

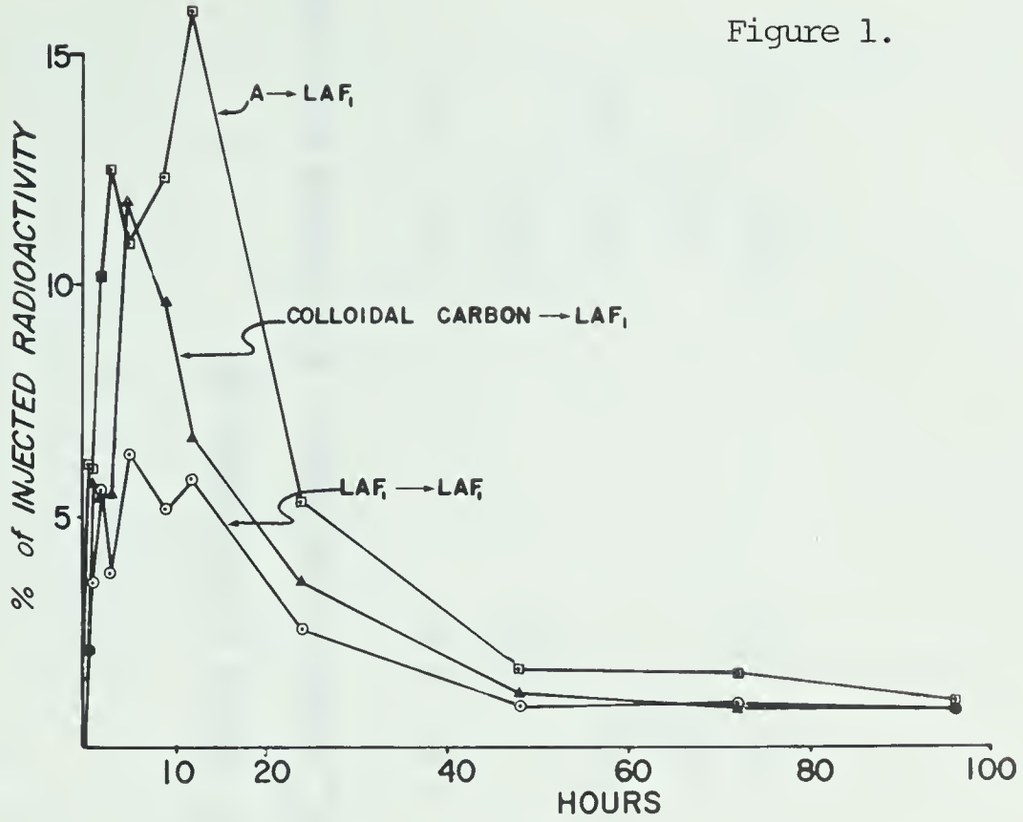

Figure 2.

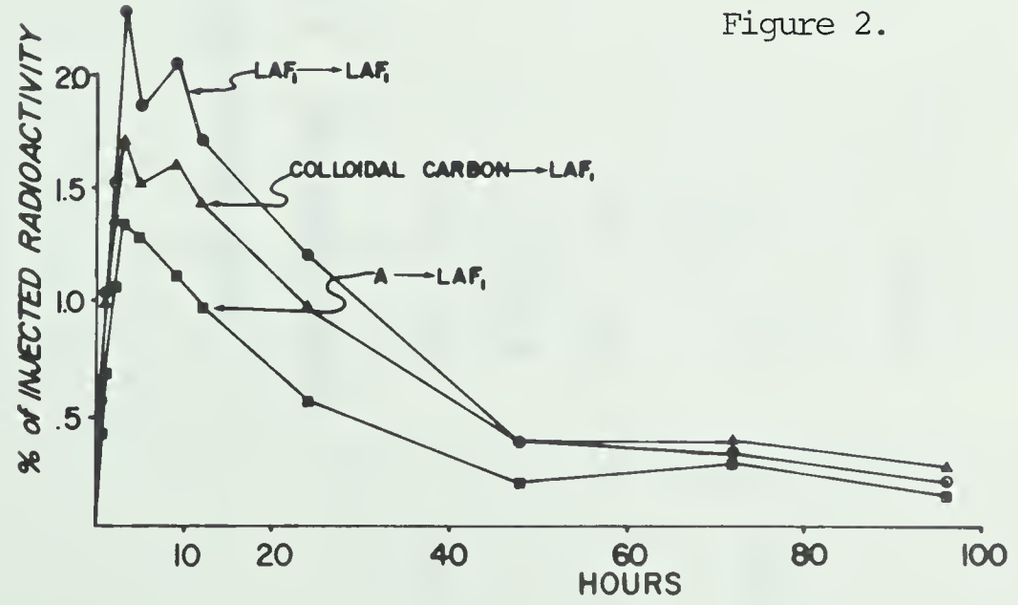

TABLE IV

SPLEEN WEIGHTS AND PEAK SPLEEN AND FEMUR Fe^{59} UPTAKES ON THE EIGHTH DAY

	Spleen weight (mg)	% Fe^{59} uptake/spleen	%Fe^{59} uptake/100 mg spleen	% Fe^{59}uptake by rt. femur
$LAF_1 \rightarrow LAF_1$	87.6 ± 13.9*	6.34 ± 2.02	7.69 ± 2.05	2.29 ± 0.12
	p<0.001	p<0.01	p<0.05	p<0.05
$A \rightarrow LAF_1$	159.7 ± 46.0	15.98 ± 4.41	7.26 ± 1.14	1.34 ± 0.62
	p>0.05	p<0.05	p<0.01	p<0.01
$C.C. \rightarrow LAF_1$	93.3 ± 16.9	11.81 ± 1.56	11.20 ± 1.22	1.71 ± 0.21

*Mean ± 1 S.D.

reveals that on a weight basis the spleens of C.C. → LAF_1 mice exhibited the greatest radioactivity uptake.

Spleen weights on the eighth day after spleen cell or colloidal carbon injection are also shown in Table IV. Mice treated with parental spleen cells developed significant splenomegaly attributed to the GVH reaction (11, 29). Colloidal carbon-treated animals revealed only slightly enlarged spleens not differing significantly from the controls.

Incorporation of radioiron into circulating erythrocytes is shown in Figures 3a and 3b. Student's t-test revealed a significant difference in the 96-hour Fe^{59} incorporation into circulating erythrocytes between control and GVH reaction animals ($p < 0.02$) but not between control and colloidal carbon-treated animals. However, incorporation into circulating erythrocytes in colloidal carbon-treated mice was significantly higher than controls at 72 hours ($p < 0.02$). Figure 3b provides a more detailed picture of the early incorporation of Fe^{59} and suggests an enhanced early radioiron utilization in both GVH and carbon-treated animals. This is in agreement with data presented for A → LAF_1 and $A^{*LAF_1} \rightarrow LAF_1$ mice by Bain in 1965 (29).

Radioiron clearance curves and the curve components, as determined by backward subtraction from both raw data and smoothed curves drawn by inspection, for the three groups of mice are presented in Figures 4a, 4b, and 4c.

Table V presents the graphically determined constants, based on both actual observed points and smoothed curves, which characterize the plasma radioiron clearance curves. For each curve the first

Figure 3a. Incorporation of radioiron into circulating erythrocytes of A → LAF_1, C.C. → LAF_1 and LAF_1 → LAF_1 mice beginning on the seventh day after spleen cell or colloidal carbon injection. Each point is the average of 3 - 5 mice.

Figure 3b. First 5 hours of Fig. 3a in greater detail showing enhanced early incorporation of Fe^{59} by A → LAF_1 and C.C. → LAF_1 animals.

Figure 3a.

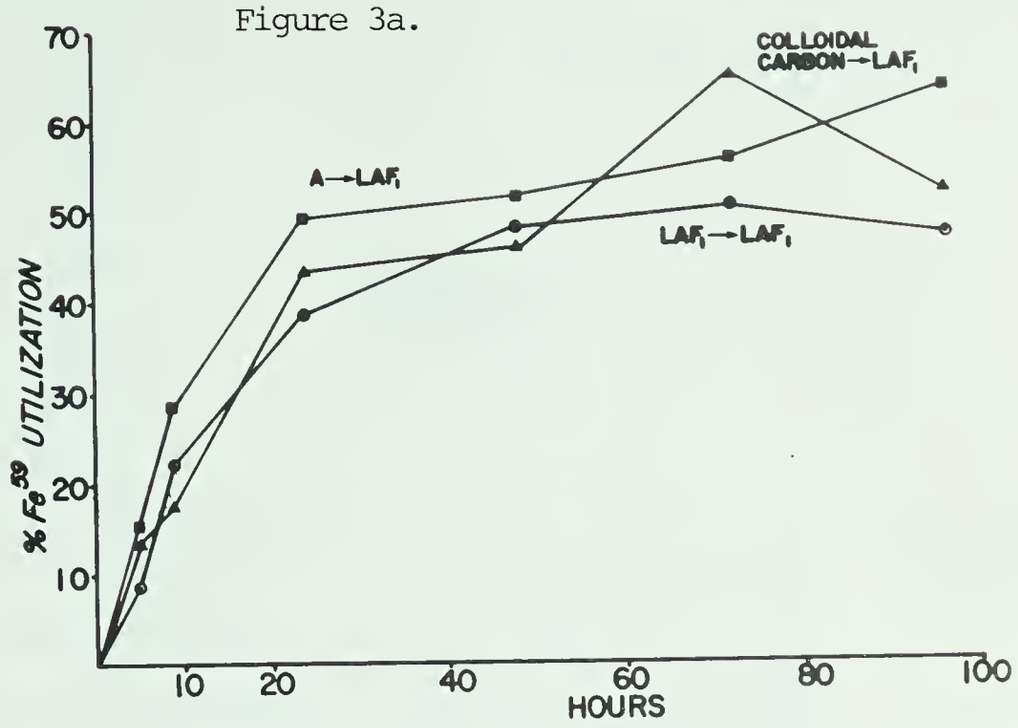

Figure 3b.

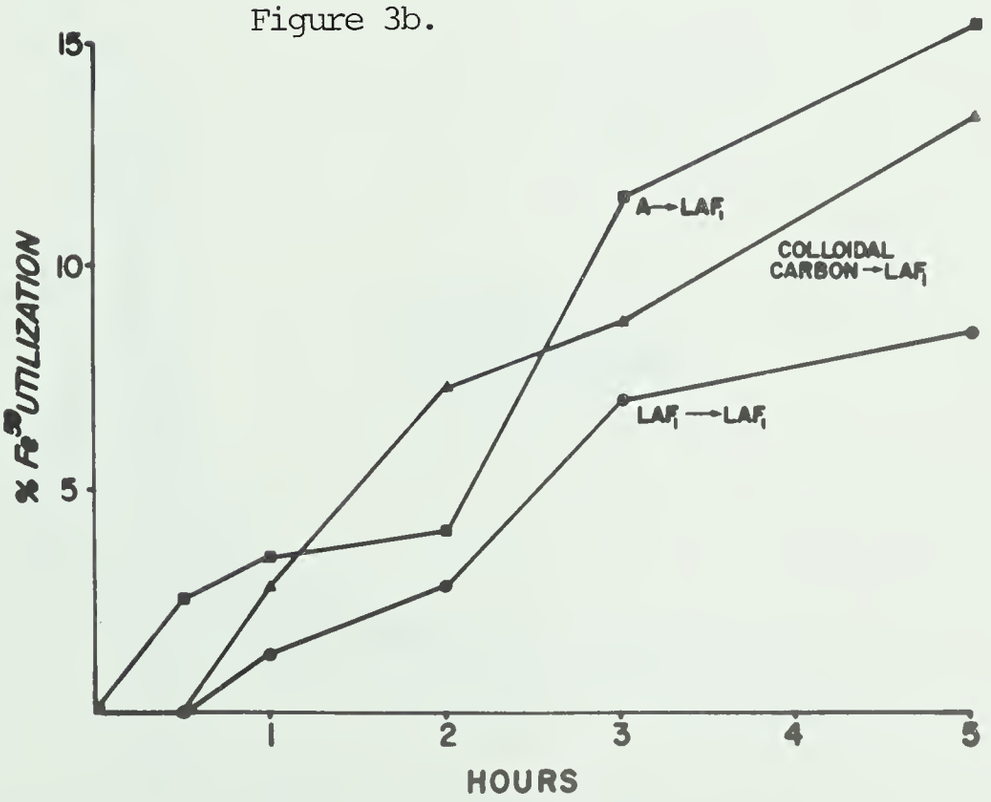

Figure 4a. Resolution of plasma radioiron clearance curve of A → LAF_1 mice into three components from actual data ($4a_1$) and from smoothed curve drawn by inspection ($4a_2$)

Figure $4a_1$.

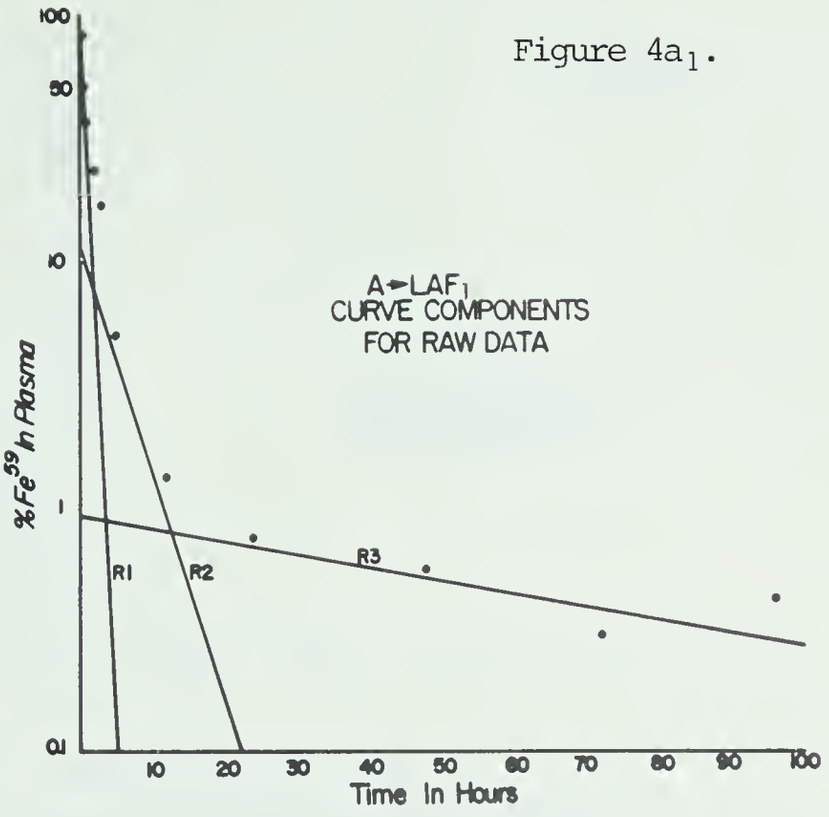

Figure $4a_2$.

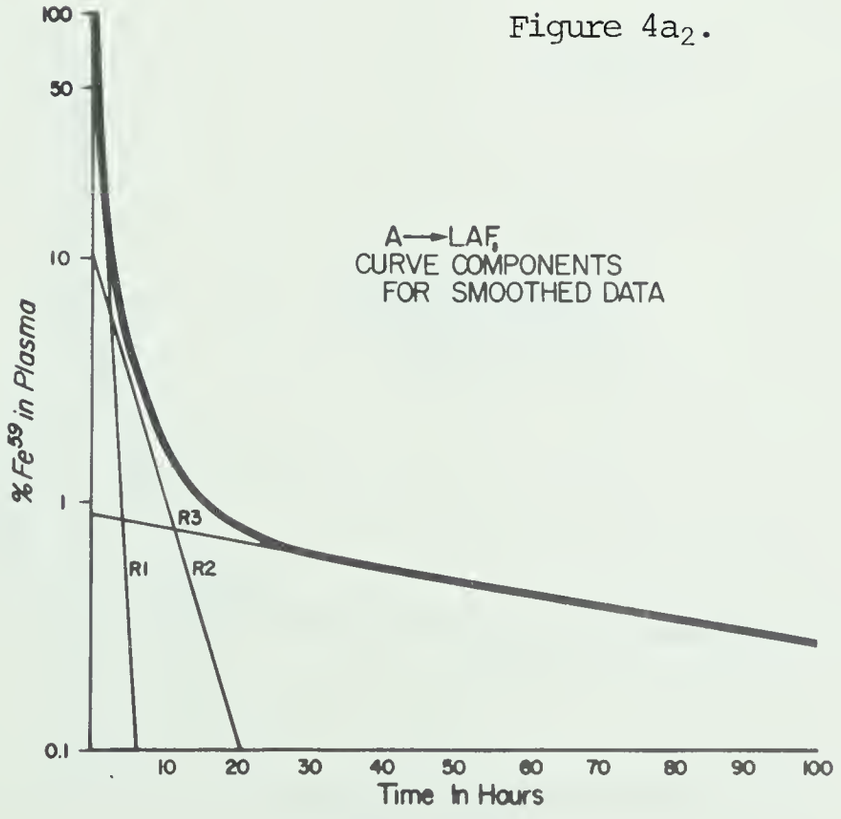

Figure 4b. Resolution of plasma radioiron clearance curve of C.C. → LAF_1 mice into three components from actual data ($4b_1$) and from smoothed curve drawn by inspection ($4b_2$).

Figure $4b_1$.

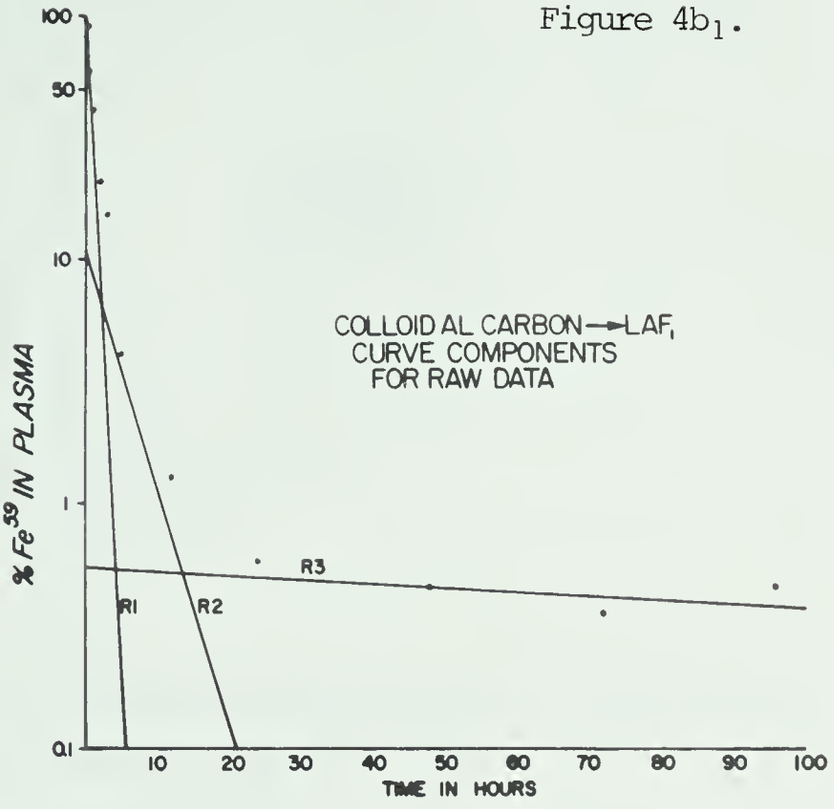

Figure $4b_2$.

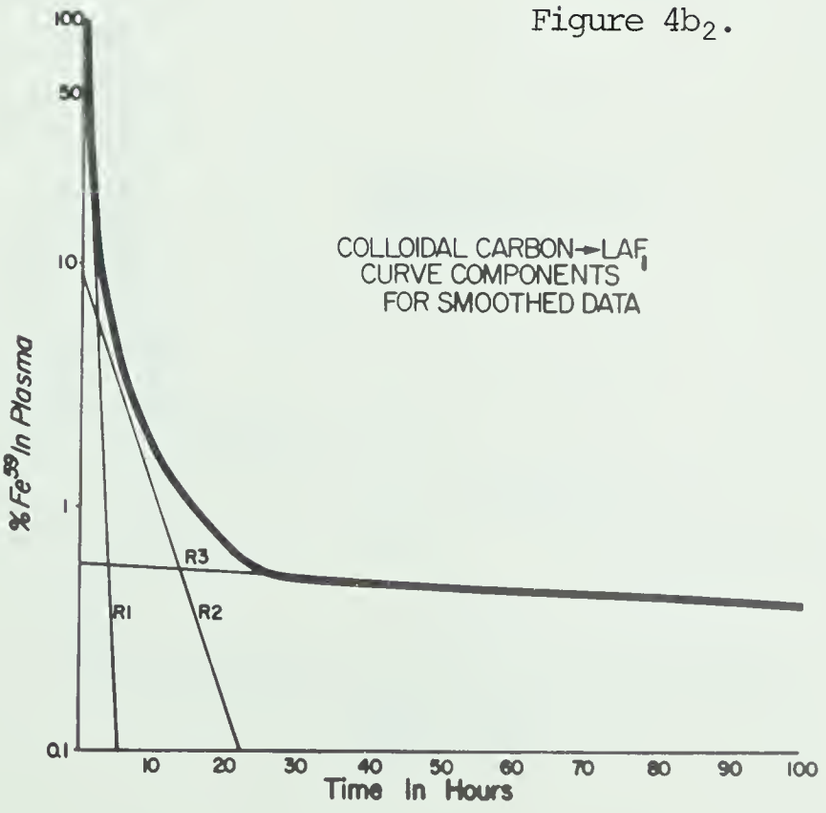

Figure 4c. Resolution of plasma radioiron clearance curve of $LAF_1 \rightarrow LAF_1$ mice into three components from actual data ($4c_1$) and from smoothed curve drawn by inspection ($4c_2$).

Figure $4c_1$.

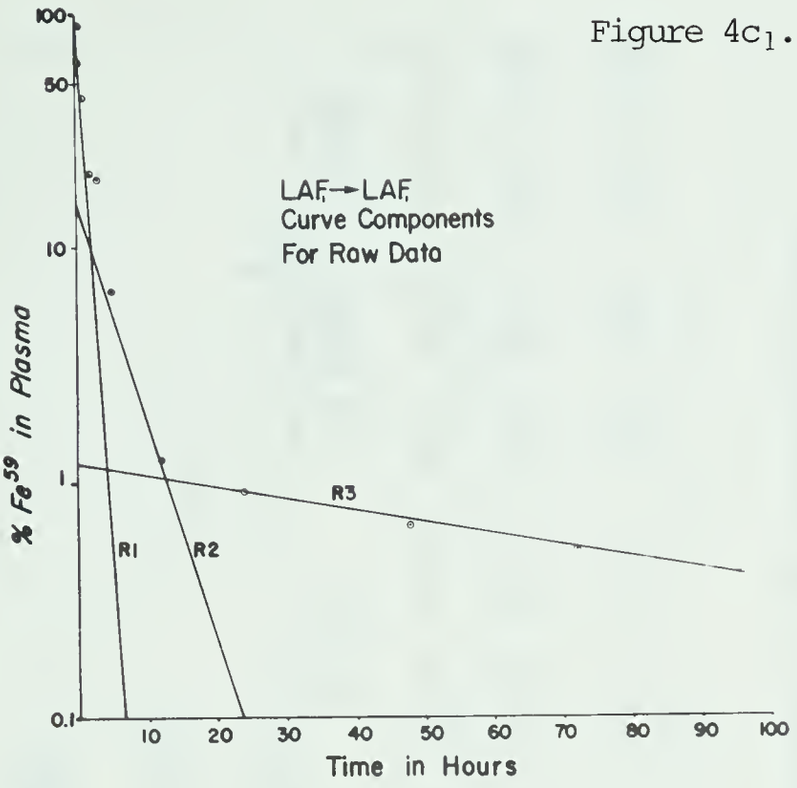

Figure $4c_2$.

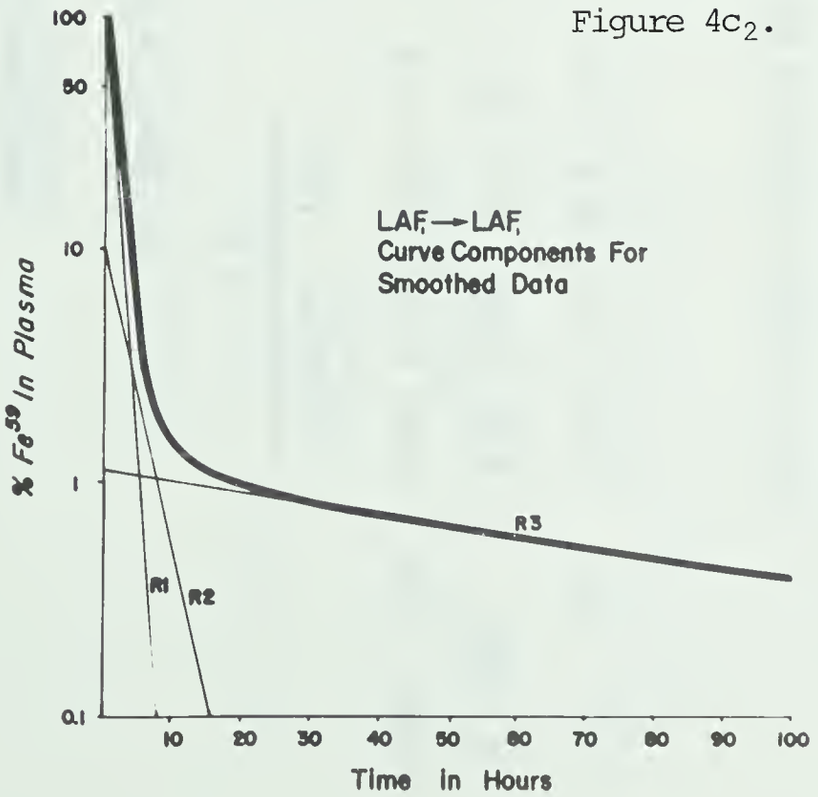

TABLE V

RADIOIRON CLEARANCE DATA

$$X = Ae^{-r_1 t} + Be^{-r_2 t} + Ce^{-r_3 t}$$

	R_1#		R_2		R_3	
	$T_{\frac{1}{2}}$ (hrs)*	A(%)*	$T_{\frac{1}{2}}$ (hrs)	B (%)	$T_{\frac{1}{2}}$ (hrs)	C (%)
LAF_1 → LAF_1	0.68, 0.76	84.0, 90.0	3.30, 2.35	15.0, 10.0	59.5, 65.0	1.20, 1.12
A → LAF_1	0.53, 0.55	88.0, 89.1	3.40, 3.15	11.0, 10.0	58.5, 58.0	0.90, 0.90
C.C. → LAF_1	0.57, 0.57	89.5, 90.1	3.15, 3.50	10.7, 9.0	>100, >100	0.55, 0.58

R_1, R_2 and R_3 indicate the three components of the clearance curves.

* First figures are based on actual observations; second on smoothed plasma radioiron clearance curves.

TABLE VI

FERROKINETIC AND HEMATOCRIT DATA

	Serum Iron (μg/100 ml)	PIT (mg/day)	Hematocrit
$LAF_1 \rightarrow LAF_1$	253 ± 83*(33)	0.059, 0.056#	46.3 ± 2.5*(33)
	$p<0.02$		
$A \rightarrow LAF_1$	210 ± 54 (31)	0.066, 0.065	45.5 ± 3.0 (32)
	$p<0.001$		
$C.C. \rightarrow LAF_1$	174 ± 88 (30)	0.051, 0.051	45.8 ± 2.7 (33)

*Mean ± 1 S.D.; figures in parenthesis are numbers of animals.

#First figures are based on actual observations; second figures are ~~are~~ based on smoothed plasma radioiron clearance curves.

component, R_1 , was interpreted as reflecting iron clearance for erythropoiesis and its $T_{\frac{1}{2}}$ was used in PIT calculations.

Ferrokinetic and hematocrit data are presented in Table VI. Significantly lower serum iron levels were found for GVH and colloidal carbon-treated animals than for control animals ($p < 0.02$ and $p < 0.001$ respectively). Hematocrit values for the two test groups, on the other hand, showed no significant decreases. PIT data suggested increased total erythropoiesis in the A → LAF_1 animals but not in C.C. → LAF_1 animals.

Histologic observations of sections of spleen supported the ferrokinetic studies in that both GVH animals and colloidal carbon-treated animals exhibited a hypercellular red pulp with an increase in erythroid precursors. The carbon was more abundant in the red pulp but small amounts were also present in the white pulp.

4. Discussion

Howard (68) showed that at the time of peak splenomegaly in the GVH reaction there is an elevated rate of clearance of colloidal carbon following intravenous administration. He interpreted this as due to an increased rate of phagocytosis as a consequence of a host response to degenerative cellular by-products of the GVH reaction. The shift of erythropoiesis from the bone marrow to the spleen during the GVH reaction may be related to the RES stimulation. The work of Fruhman (60 - 62), who observed such a shift in mice upon stimulation of the RES with endotoxin, zymosan, or starvation and re-feeding, is compatible with this hypothesis. Fruhman suggested that the increased erythropoiesis in the

spleen compensates for the decrease in the marrow and that as a result there is no change in the total erythropoiesis. However, he did not measure total erythropoiesis.

Plasma iron turnover is regarded as a measure of total erythropoiesis (73). Evidence from the present study suggests that, following stimulation of the murine RES with colloidal carbon, total erythropoiesis, as measured by the PIT, is unchanged. Colloidal carbon in the dosage employed did, however, cause an erythropoietic shift, although this shift was not as pronounced as that caused by the injection of parental spleen cells. A previous study (29) showed increases in total erythropoiesis in the GVH reaction greater than those observed in the present experiments but the pattern of the erythrokinetic changes was the same. RES stimulation by colloidal carbon does not per se reproduce the ferrokinetic changes of the GVH reaction. The cause of the erythropoietic shift in animals undergoing the GVH reaction and in colloidal carbon-treated animals remains obscure. Its mechanism is examined in following sections.

V. EFFECT OF SPLENECTOMY ON THE FERROKINETICS OF THE GVH REACTION

1. Introduction

The prominence of the spleen in mouse and rat erythropoiesis has been well documented (58, 60-65, 76). Results presented in Chapter IV and elsewhere (29) showed that the GVH reaction, due to the injection of parental strain spleen cells into LAF_1 mice, has two contrasting erythropoietic effects: (1) splenic erythropoiesis is stimulated, and (ii) bone marrow erythropoiesis is depressed. The question of the relationship between these two manifestations of the GVH reaction in LAF_1 mice prompted a ferrokinetic study in mice splenectomized seven days before receiving the parental spleen cell inoculation. It was thereby hoped to show whether the erythropoietic depression observed in the marrow, as measured by Fe^{59} uptake, was dependent upon concurrent changes in the spleen.

2. Results

Results of six-hour radioactivity levels in the spleen, right femur, circulating erythrocytes, serum, liver and tail on the eighth day of the GVH reaction are presented in Table VII. The salient findings were:

(i) The usual increase in the uptake of $Fe^{59}$by the spleens of sham-splenectomized GVH animals was observed.

(ii) Splenectomized GVH mice exhibited a depression in marrow erythropoiesis (as compared with controls,Group II) similar to that observed in sham-splenectomized GVH mice, but the degree of depression

TABLE VII

EFFECT OF SPLENECTOMY ON 6-HOUR RADIOACTIVITY LEVELS# ON THE EIGHTH DAY OF THE GVH REACTION

Group	Spleen	Rt. Femur	Circ. RBC	Serum	Liver (per 100 mg)	Tail (per 1 inch)
	n = 9	n = 9	n = 9	n = 9	n = 5	n = 5
A → splen. LAF_1 (I)	-	1.59 ± 0.38	15.64 ± 4.06	5.68 ± 1.89	1.19 ± 0.32	0.50 ± 0.08
		p<0.001				
LAF_1 → splen. LAF_1(II)	-	2.46 ± 0.31	14.98 ± 3.16	4.21 ± 1.60	1.22 ± 0.19	0.49 ± 0.07
		p<0.01	p<0.05	p<0.01		
A → sham-splen. LAF_1(III)	14.61 ± 4.35*	1.03 ± 0.34	21.21 ± 5.22	3.47 ± 1.18	0.98 ± 0.12	0.56 ± 0.07
	p<0.001	p<0.001	p<0.01	p<0.01	p<0.02	
LAF_1 → sham-splen. LAF_1(IV)	6.90 ± 1.33	2.14 ± 0.35	12.66 ± 3.81	5.10 ± 0.85	1.34 ± 0.18	0.58 ± 0.11

*Mean ± 1 S.D.; # % of injected dose.

was not as pronounced as in the latter.

(iii) A → splen. LAF_1 mice did not show the increase in uptake of Fe^{59} in circulating erythrocytes shown by the A → sham-splen. LAF_1 mice when compared with controls.

(iv) Residual radioactivity in the serum of A → splen. LAF_1 mice was not significantly different from controls whereas sham-splenectomized GVH animals exhibited significantly lower serum radioactivity levels when compared with controls (Group IV).

(v) The radioactivity levels in the livers of Group I mice were not significantly different from the controls (Group II) whereas Group III mice showed significantly lower levels than their controls (Group IV).

(vi) The four groups of mice showed no significant differences in the radioactivity levels in the tail.

3. Discussion

It was shown by Biozzi et al. in 1964 (77) that splenectomy before injection of parental strain spleen cells caused some amelioration in the GVH reaction. These authors supported the suggestion of Michie and Woodruff (78) that the attenuation was due to the failure of many donor spleen cells to gain a foothold when injected into splenectomized animals. Splenectomy removes a major site to which these cells home and, hence, a lessening in the severity of the resulting GVH reaction might be anticipated. Radioactivity levels in the circulating erythrocytes, serum and livers of A → splen. LAF_1 mice would indicate that this attenuation has indeed occurred.

In spite of the ameliorating effect of splenectomy, the erythropoietic activity in the bone marrow of splenectomized animals undergoing the GVH reaction was depressed. This is an indication that the changes occurring in the marrow are not strictly dependent upon the presence of the spleen.

Radioactivity levels in the tails of the four groups of mice showed that there was no extension of erythropoiesis to this site due to the absence of the spleen and/or due to the GVH reaction. The diminished residual hepatic blood radioactivity in sham-splenectomized GVH animals confirms the findings of Bain (45) who suggested that this may be due to a decrease in the liver sinusoidal volume due to encroachment by hypertrophied and hyperplastic hepatocytes and Kupffer cells. Another possible explanation would be decreased radioactivity of hepatic lymph due to increased radioiron uptake in other sites.

Further studies on the nature of the relationship between marrow and splenic erythropoiesis during the GVH reaction are presented in Chapter VI.

VI. AUTORADIOGRAPHIC STATHMOKINETIC STUDIES

1. Introduction

Investigations thus far on the nature of the erythropoietic shift have established two points: (i) the shift from the bone marrow to the spleen may be induced by stimulation of the reticulo-endothelial system, and (ii) erythropoietic depression in the marrow is not strictly dependent upon concurrent changes in the spleen. In this section the mechanism of the shift is examined. The objectives of the experiment were to gather information regarding the following:

(i) quantitative data on the degrees of erythropoietic change in the spleen and marrow;

(ii) mitotic activity of labeled (radioiron incorporating, erythropoietic) and unlabeled (non-radioiron incorporating) cells in the spleen and marrow;

(iii) diameters of labeled cells in the two organs.

2. Combined colchicine - Fe^{59} autoradiography technique

The mitosis-arresting property of the alkaloid colchicine was first described many years ago by Pernice (79). One of the first authors applying colchicine to the study of cell kinetics was Brues in 1936 (80). Since that time it has been applied successfully in the study of many cell renewal systems. The mechanism of colchicine inhibition of mitosis has been studied by Taylor (54). By the use of tritiated colchicine he showed that colchicine is reversibly bound

to a set of sites within the cell, and that mitosis will be arrested if a critical fraction of these sites (3 - 5%) is occupied by colchicine because the cell is then unable to form a functional mitotic spindle.

The combined stathmokinetic - Fe^{59} autoradiography procedure is a new technique which permits the identification of erythropoietic cells in mitosis by virtue of the fact that Fe^{59} uptake is specific for cells actively synthesizing hemoglobin.

3. Results

In calculating the mitotic index of labeled cells in the bone marrow of $A^{*LAF_1 \rightarrow LAF_1}$ mice one value was discarded because it was greater than two standard deviations from the mean. Absolute numbers for the different cell types were calculated from the total nucleated cell counts determined at the time of cell suspension and the percentages observed for the various types in the microscopic examination of the autoradiographic smears. Figure 5 illustrates the general appearance of typical autoradiographs.

a. Bone Marrow

The numbers of the various nucleated cell types in the pooled marrow of both femurs in the control and GVH reaction animals are recorded in Tables VIIIA and VIIIB. The main findings in $A^{*LAF_1 \rightarrow LAF_1}$ mice were:

(i) a significant decrease in the number of nucleated cells due in large part to the decrease in lymphocytes;

Figure 5. Microphotographs of autoradiographic smears with (i) silver grains in focus, and (ii) cells in focus. Wright's stain. Original magnification 1250 x.

a & b. Spleen cell smear from $A^{*LAF_1 \rightarrow LAF_1}$ mouse showing a colchicine-metaphase with 16 grains. To the left and touching the mitotic figure is an eosinophil; to the right and below are lymphocytes.

c & d. Bone marrow smear from $LAF_1 \rightarrow LAF_1$ mouse showing a large cell in metaphase displaying 21 grains.

e & f. Spleen cell smear from $LAF_1 \rightarrow LAF_1$ mouse showing a heavily labeled cell (at least 20 silver grains) surrounded by lymphocytes. Note the characteristic scatter of grains about the cell.

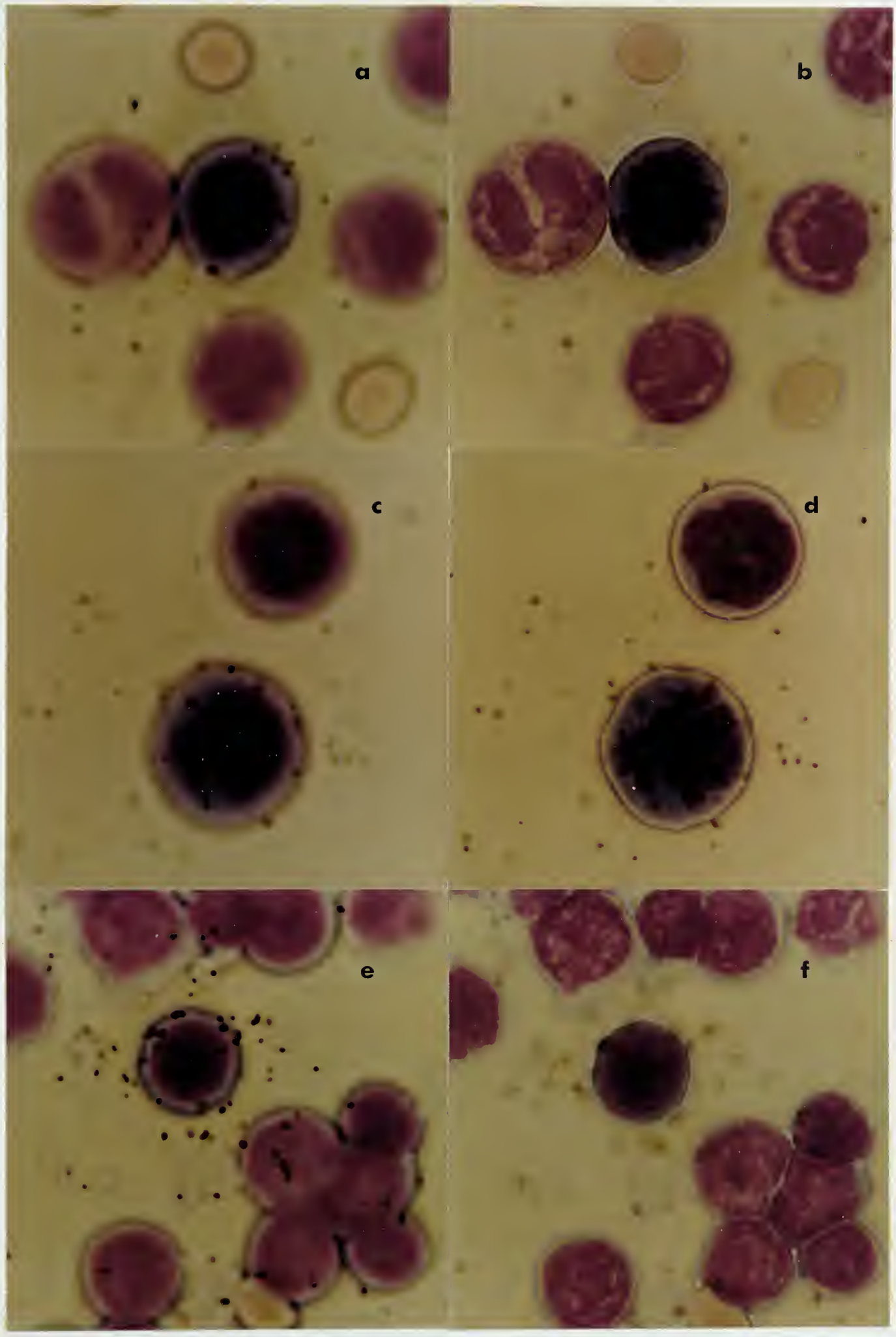
a
b
c
d
e
f

TABLE VIIIA

NUMBERS OF CELLS IN THE BONE MARROW OF $LAF_1 \rightarrow LAF_1$ AND $A^{*LAF_1 \rightarrow LAF_1}$ MICE ON THE EIGHTH DAY OF THE GVH REACTION

	$LAF_1 \rightarrow LAF_1$ (21)		$A^{*LAF_1 \rightarrow LAF_1}$ (22)
Total number nucleated cells	$24.60 \pm 6.34 \times 10^{6}$*	$p < 0.001$	$15.60 \pm 4.99 \times 10^{6}$
Lymphocytes	8.52 ± 1.28	$p < 0.001$	2.65 ± 0.67
Segmented and band neutrophils	7.80 ± 1.10	$p > 0.05$	8.31 ± 0.95
Eosinophils	1.02 ± 0.30	$p < 0.001$	0.78 ± 0.31
Myelocytes and metamyelocytes	1.12 ± 0.60	$p < 0.01$	1.69 ± 0.69
Unlabeled blast cells	0.33 ± 0.15	$p > 0.05$	0.33 ± 0.13
Unidentified, unlabeled cells	0.10 ± 0.10	$p > 0.05$	0.10 ± 0.08
Labeled cells	4.38 ± 1.08	$p < 0.001$	0.97 ± 0.43
Unlabeled mitotic figures	1.31 ± 0.47	$p < 0.001$	0.78 ± 0.35
Labeled mitotic figures	1.16 ± 0.42	$p < 0.001$	0.20 ± 0.13
$\frac{\text{Polychromatic rbc}}{\text{mature rbc}}$	0.30 ± 0.22	$p < 0.001$	0.08 ± 0.06

*Mean ± 1 S.D.; two femurs pooled. Figures in parenthesis are numbers of animals.

TABLE VIIIB

RELATIVE NUMBERS OF CELLS IN THE BONE MARROW OF $LAF_1 \rightarrow LAF_1$ AND $A^*{}^{LAF_1 \rightarrow LAF_1}$ MICE ON THE EIGHTH DAY OF THE GVH REACTION

	$LAF_1 \rightarrow LAF_1$ (21)	p	$A^*{}^{LAF_1 \rightarrow LAF_1}$ (22)
Lymphocytes	34.63 ± 5.31%*	<0.001	16.99 ± 4.31%
Segmented and band neutrophils	31.73 ± 4.47	<0.001	53.26 ± 6.16
Eosinophils	4.16 ± 1.20	>0.05	5.04 ± 2.00
Myelocytes and metamyelocytes	4.55 ± 2.45	<0.001	10.84 ± 3.81
Unlabeled blast cells	1.33 ± 0.58	<0.01	1.94 ± 0.80
Unidentified, unlabeled cells	0.43 ± 0.42	>0.05	0.67 ± 0.52
Labeled cells	17.81 ± 4.39	<0.001	6.24 ± 2.77
Unlabeled mitotic figures	5.47 ± 1.91	>0.05	5.01 ± 2.24
Labeled mitotic figures	4.71 ± 1.65	<0.001	1.40 ± 0.87

*Mean ± 1 S.D.; two femurs pooled. Figures in parenthesis are numbers of animals.

(ii) a significant increase in myelocytes and metamyelocytes;

(iii) a four-fold decrease in the number of labeled (erythropoietic) cells;

(iv) a three- to four-fold decrease in the ratio of polychromatic red blood cells to mature red blood cells.

Table IX shows the mitotic indices and turnover times, calculated as described on page 34, of labeled and unlabeled cells in the marrows of control and test mice. A decrease in the overall mitotic index and in the mitotic index of labeled cells was observed in GVH animals. The decreased mitotic index of labeled cells was reflected in the increased turnover time for these cells.

Mean diameters of labeled marrow cells in GVH mice were found to be significantly less than controls (Table XII).

b. Spleen

Data on cell numbers, mitotic indices and turnover times, and labeled cell diameters are presented in Tables XA, XB, XI, and XII respectively. The important findings in the GVH reaction were:

(i) a significant increase in the total number of nucleated cells;

(ii) a significant decrease in the number of lymphocytes;

(iii) an eight-fold increase in the numbers of myelocytes and metamyelocytes;

(iv) a four-fold increase in the number of plasma cells;

(v) a tripling of unlabeled blast cells;

TABLE IX

MITOTIC INDICES AND TURNOVER TIMES OF LABELED AND UNLABELED CELLS IN BONE MARROW OF $LAF_1 \rightarrow LAF_1$ AND $A^{*LAF_1 \rightarrow LAF_1}$ ANIMALS

	$LAF_1 \rightarrow LAF_1$	p	$A^{*LAF_1 \rightarrow LAF_1}$
	(21)		(22)
Mean 6 hour overall M.I.	10.04 ± 2.91%*	<0.001	6.41 ± 2.40%
Mean 6 hour M.I. of labeled cells	26.35 ± 6.16%	=0.05	21.48 ± 9.21%
Mean T.T. of labeled cells	23 hours		28 hours
Mean 6 hour M.I. of unlabeled cells	6.53 ± 2.46%	>0.05	5.32 ± 2.24%
Mean T.T. of unlabeled cells	92 hours		113 hours

*Mean ± 1 S.D.; M.I. = mitotic index; T.T. = turnover time. Figures in parenthesis are numbers of animals

TABLE XA

NUMBERS OF CELLS IN THE SPLEENS OF $LAF_1 \rightarrow LAF_1$ AND $A^{*LAF_1 \rightarrow LAF_1}$ ANIMALS ON THE EIGHTH DAY OF THE GVH REACTION

	$LAF_1 \rightarrow LAF_1$ (21)	p	$A^{*LAF_1 \rightarrow LAF_1}$ (22)
Total number nucleated cells	$18.47 \pm 3.02 \times 10^7$*	<0.001	$22.30 \pm 2.84 \times 10^7$
Lymphocytes	16.85 ± 0.43	<0.001	15.79 ± 1.01
Segmented and band neutrophils	0.83 ± 0.26	<0.001	2.42 ± 0.56
Eosinophils	0.07 ± 0.06	>0.05	0.10 ± 0.07
Myelocytes and metamyelocytes	0.03 ± 0.04	<0.001	0.25 ± 0.14
Plasma cells	0.02 ± 0.02	<0.001	0.10 ± 0.08
Unlabeled blast cells	0.16 ± 0.14	<0.001	0.52 ± 0.27
Unidentified, unlabeled cells	0.00	-	0.03 ± 0.05
Labeled cells	0.49 ± 0.19	<0.001	2.82 ± 0.88
Unlabeled mitotic figures	0.04 ± 0.03	<0.001	0.26 ± 0.13
Labeled mitotic figures	0.06 ± 0.03	<0.001	0.34 ± 0.11
$\frac{\text{Polychromatic rbc}}{\text{mature rbc}}$	0.06 ± 0.01	<0.001	0.10 ± 0.03

*Mean ± 1 S.D. Figures in parenthesis indicate numbers of animals.

TABLE XB

RELATIVE NUMBERS OF CELLS IN THE SPLEENS OF $LAF_1 \rightarrow LAF_1$ AND $A^{*LAF_1 \rightarrow LAF_1}$ ANIMALS ON THE EIGHTH DAY OF THE GVH REACTION

	$LAF_1 \rightarrow LAF_1$ (21)	p	$A^{*LAF_1 \rightarrow LAF_1}$ (22)
Lymphocytes	91.10 ± 2.35%*	<0.001	70.80 ± 4.65%
Segmented and band neutrophils	4.47 ± 1.41	<0.001	10.84 ± 2.52
Eosinophils	0.36 ± 0.34	>0.05	0.46 ± 0.32
Myelocytes and metamyelocytes	0.17 ± 0.22	<0.001	1.17 ± 0.73
Plasma cells	0.11 ± 0.03	<0.001	0.47 ± 0.08
Unlabeled blast cells	0.88 ± 0.73	<0.001	2.35 ± 1.22
Unidentified, unlabeled cells	0.00		0.12 ± 0.22
Labeled cells	2.66 ± 1.02	<0.001	12.63 ± 3.95
Unlabeled mitotic figures	0.23 ± 0.14	<0.001	1.15 ± 0.59
Labeled mitotic figures	0.34 ± 0.19	<0.001	1.51 ± 0.51

*Mean ± 1 S.D. Figures in parenthesis indicate numbers of animals.

TABLE XI

MITOTIC INDICES AND TURNOVER TIMES OF LABELED AND UNLABELED CELLS IN SPLEENS OF $LAF_1 \rightarrow LAF_1$ AND $A^{*LAF_1} \rightarrow LAF_1$ MICE ON THE EIGHTH DAY AFTER SPLEEN CELL INJECTIONS

	$LAF_1 \rightarrow LAF_1$ (21)	p	$A^{*LAF_1} \rightarrow LAF_1$ (22)
Mean 6 hour overall M.I.	0.58 ± 0.24%*	<0.001	2.66 ± 0.74%
Mean 6 hour M.I. of labeled cells	13.37 ± 5.55%	>0.05	12.44 ± 3.55%
Mean T.T. of labeled cells	45 hours		48 hours
Mean 6 hour M.I. of unlabeled cells	0.24 ± 0.14	<0.001	1.32 ± 0.64%
Mean T.T. of unlabeled cells	104 days		19 days

*Mean ± 1 S.D.; M.I. = mitotic index; T.T. = turnover time.
Figures in parenthesis indicate number of animals.

TABLE XII

SIZE OF LABELED CELLS IN BONE MARROW AND SPLEEN OF CONTROL AND GVH ANIMALS ON THE EIGHTH DAY AFTER SPLEEN CELL TRANSFER

	Bone Marrow		Spleen
$LAF_1 \rightarrow LAF_1$	8.49 ± 0.12*μ	$p < 0.001$	7.71 ± 0.18
	$p < 0.01$		$p > 0.05$
$A^{*LAF_1 \rightarrow LAF_1}$	7.78 ± 0.34	$p > 0.05$	7.60 ± 0.26

*Mean diameter in μ ±1 S.D.

(vi) a five-fold increase in the number of labeled cells;

(vii) a significant increase in the ratio of polychromatic red blood cells to mature red blood cells;

(viii) a significant increase in the overall mitotic index due to increased mitotic activity of unlabeled cells;

(ix) no significant change in the mitotic index of labeled cells;

(x) no significant change in the mean diameters of labeled cells.

4. Interpretation and Discussion

Lymphoid depletion as a consequence of the GVH reaction has been reported by many authors (14, 27, 38, 39). The pronounced decrease in the number of lymphocytes in both bone marrow and spleen observed in the present study confirms this finding. Accompanying the lymphocyte depletion was an increase in the numbers of myelocytes and metamyelocytes. The increase in the number of plasma cells (from 0.1% to 0.5%) observed in the spleen is a reflection of the immunological nature of the GVH reaction.

The major bone marrow findings in the GVH reaction with respect to labeled (erythropoietic) cells were:

(i) a decrease in the mean diameter of labeled cells;

(ii) a decrease in the number of labeled cells;

(iii) a decrease in the mitotic index of labeled cells.

These findings may be the result of any one of the following:

(i) arrest of proliferation with continued maturation;

(ii) selective emigration of larger mitotically active cells;

(iii) arrest of proliferation of large cells and emigration of the more mature cells;

(iv) emigration of large (primitive) and small (mature) cells but more of the former.

The main spleen findings, with respect to erythropoietic cells, in the GVH reaction were:

(i) no change in mean cell diameter;

(ii) an increase in number of labeled cells;

(iii) no change in the mitotic index of labeled cells.

These findings would be compatible with either of the following:

(i) the increase in number could be due to immigration of cells;

(ii) the increase in cells, unaccompanied by a change in diameter or mitotic index, could be the result of a prior influx of large mitotically active cells which have matured by the time of examination. This explanation seems unlikely because the study was done before the peak of splenic erythropoiesis had been reached.

Explanation (i) is the most likely with respect to the spleen. The only bone marrow interpretation with which it is entirely compatible is (iii). No. (iv) might theoretically be compatible but it seems unlikely because it poses two difficult requirements: (i) that primitive cells have a greater tendency to leave the bone marrow than mature cells, and (ii) that such primitive cells leaving the bone marrow do not settle in the spleen but mature somewhere else. However, no other sites of erythropoiesis have been found in these animals (Table VII) and (29).

Bain in 1964 (81) found, on the ninth day after spleen cell injection, a four- to five-fold increase in the number of normoblasts in peripheral blood of $A^{*LAF_1} \rightarrow LAF_1$ mice. Bone marrow explanation no. (iii), taken together with spleen explanation no. (i), is compatible with this movement of immature erythropoietic cells into the circulating blood. It is also compatible with the early direct uptake of radioiron into circulating erythrocytes (29).

Thus, the following mechanism may be postulated to fit the available data on the erythropoietic shift:

(i) Arrest of proliferation of erythroid precursors in the marrow.

(ii) Migration of the more mature erythropoietic (labeled) cells from the marrow to the spleen.

VII. SUMMARY AND GENERAL DISCUSSION

1. Summary

Studies on the nature of the erythropoietic shift from the bone marrow to the spleen in the GVH reaction in F_1 hybrid mice, due to the intraperitoneal injection of parental spleen cells, led to the following findings:

(i) A comparative study showed that the shift can be brought about by stimulation of the reticulo-endothelial system by the intravenous administration of colloidal carbon at a dose of 16 mg per 100 gm body weight. It differs from the shift caused by the injection of parental spleen cells in that it is not accompanied by splenomegaly and increased plasma iron turnover.

(ii) LAF_1 mice splenectomized seven days prior to the parental spleen cell injection exhibited a depression in marrow erythropoiesis. However, it was not as pronounced as in sham-splenectomized GVH mice.

(iii) Autoradiographic stathmokinetic studies revealed:

(a) a four-fold decrease in the number of labeled cells in the marrow and a five-fold increase in the spleen;

(b) a decrease in the mitotic index of labeled cells in the marrow with no change in the spleen;

(c) a reduction in the mean labeled cell diameter in the marrow with no significant change in the spleen;

(d) a pronounced decrease in the number of lymphocytes accom-

panied by an increase in the number of myelocytes and metamyelocytes in both marrow and spleen;

(e) a four-fold increase in the number of plasma cells in the spleen.

2. General Discussion

It has been shown in rats that a severe erythropoietic depression, such as that produced by continuous whole-body irradiation (70R per day for 7 - 8 weeks) or a combination of radiation and phenylhydrazine (64, 67), causes marked splenic erythropoiesis, after five to six weeks of exposure, without concomitant bone marrow erythropoiesis. Evidence from the present study on mice indicated that the spleen may supplant the bone marrow as the major site of erythropoiesis in the body even though there is little or no anemia. Thus, it would appear that erythropoiesis in the marrow is more susceptible to depression by radiation or GVH reaction than erythropoiesis in the spleen.

A link between the erythropoietic shift of the GVH reaction and RES stimulation was postulated in Chapter IV. However, it was shown that stimulation of the RES with colloidal carbon does not reproduce all of the ferrokinetic changes of the GVH reaction. This suggests that RES stimulation alone is probably not responsible for all of the erythropoietic and ferrokinetic alterations observed. On the other hand, this possibility cannot be discounted entirely since the observed differences might be attributable to different degrees of RES stimulation. Responses to varying degrees of RES stimulation were not

investigated.

Evidence provided by the splenectomy experiment revealed that the erythropoietic depression in the marrow in the GVH reaction can occur in the absence of the spleen. On the other hand, stathmokinetic studies indicated a migration of erythropoietic cells from the marrow to the spleen as a partial explanation for the shift, with a decrease in the mitotic activity of bone marrow erythropoietic cells also contributing. Presumably therefore, in the absence of the spleen, nucleated erythroid precursors may go to other sites or remain longer in the blood.

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